Neutralisation of influenza virus : evidence that neutralisation by IgG is the result of incomplete uncoating rather than a reduction in the virion transcriptase activity.

by

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To my Mum and Dad,

my brothers Simonjohn, Nicolas and Andrew

(and everyone who knows me!)

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Declaration

This thesis has been composed by myself and has not been accepted in any previous application for a degree. The work presented was done by myself and all sources of materials and information have been acknowledged.

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SUMMARY

This thesis is an account of experiments designed to investigate further the mechanism of neutralisation of influenza virus by immunoglobulin G (IgG).

Section III demonstrates that neutralised virus does not direct primary transcription *in vivo* thus extending the findings of Possee *et al*. (1982) who reported lack of detectable secondary transcription.

Evidence is presented in Section IV which suggests an explanation for the lack of transcription *in vivo*. Neutralised virus undergoes incomplete uncoating such that the vRNA does not become susceptible to RNase digestion. However, consistent with the findings of other workers, IgG-neutralised virus attached to cells and the vRNA became localised in the nucleus to the same extent as infectious virus. It is therefore proposed that neutralisation of influenza virus by IgG is the result of failure to uncoat sufficiently for the virion transcriptase enzyme to become active.

The transcriptase activity of neutralised influenza virus was also investigated *in vitro* using mRNA as primer. None of the cap-utilising functions were significantly inhibited (Section II). This suggests that not all neutralising antibodies cause the inhibition of transcription *in vitro*, first described by Possee *et al*. (1982). It is probable that disruption of the virus membrane *in vitro* does not accurately mimic the uncoating processes that take place *in vivo*.

ABBREVIATIONS

ApG	Adenylyl (3'-5') guanosine
BAP	Bacterial alkaline phosphatase
BHA	Bromelain-released haemagglutinin
BHK-21	Baby hamster kidney
bis-acrylamide	N-N'-methylene bisacrylamide
CEF	Chick embryo fibroblast
cRNA	Complementary RNA
cpm	counts per minute
CYH	cycloheximide
CPV	Cytoplasmic polyhedrosis virus, type 1
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
F	Fusion
Fab	Fragment antigen binding (Papain digestion product)
$F(ab')_2$	Divalent antigen binding fragment (Pepsin digestion product)
Fc	Fragment crystallisable
FPV	Fowl plague virus
FPV/R	Fowl plague virus/Rostock
G	Glycoprotein
GMEM	Glasgow modified Eagle's medium
HA	Haemagglutinin
HAU	Haemagglutinin units
Ig	Immunoglobulin
M	Matrix
MDCK	Madin-Darby canine kidney

m^7G	7-methyl guanosine
moi	Multiplicity of infection
mRNA	Messenger RNA
NA	Neuraminidase
NCS	Newborn calf serum
NML	Nuclear monolayer
NP	Nucleoprotein
P	Polymerase
PA	Influenza virus RNA segment 3 gene product (polymerase protein)
PAGE	Polyacrlamide gel electrophoresis
PB1	Influenza virus RNA segment 2 gene product (polymerase protein)
PB2	Influenza virus RNA segment 1 gene product (polymerase protein)
PBS	Phosphate buffered saline
pfu	plaque forming units
p.i.	Post-infection
pIg	Pre-immunisation Ig
poly(A)	polyadenylated
RAM-Fab	Rabbit anti-mouse IgG, Fab specific
Reovirus 3	Reovirus type 3, Dearing strain
RNA pol II	RNA polymerase II
RNase	Ribonuclease
RNP	Ribonucleoprotein
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
TN	Tris-sodium chloride
TNE	Tris-sodium chloride-EDTA

Tris	Tris(Hydroxymethyl) aminomethane
ts	Temperature-sensitive
vRNA	viral RNA

GENERAL INTRODUCTION

INFLUENZA VIRUS STRUCTURE AND FUNCTION

1. INFLUENZA VIRUSES

Influenza viruses are divided into types A, B and C on the basis of the antigenicity of the nucleoprotein (NP) (the type-specific antigen). The viral genome is single-stranded RNA, cannot be translated and is therefore said to be of negative polarity. Later sequence data showed that this was complementary to the message. The genome is segmented: A and B type viruses have 8 segments and C type viruses have 7 segments. Reassortment of segments occurs between viruses of the same type (Tobita and Kilbourne, 1974; Racianello and Palese, 1979) but reassortment between types has not been demonstrated: cells doubly infected with A and B type viruses produce phenotypically ' ed virions with nucleocapsids from a single parent (Sklyanskaya, et al., 1985). A and B type viruses have two envelope glycoproteins but Type C viruses have only one. Type A and B influenza viruses are so similar that they can be considered as comprising one sub-group and type C viruses another.

In the present study only type A influenza viruses were used and therefore B and C type viruses will not be considered further.

2. MORPHOLOGY OF INFLUENZA VIRIONS

The morphology of influenza virus as seen under the electron microscope (reviewed by Wrigley, 1979) varies with passage

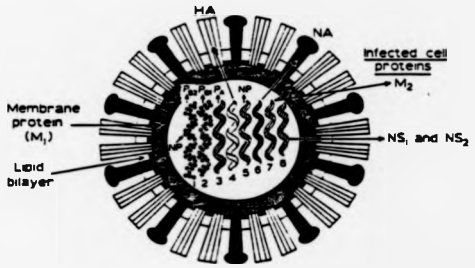


Figure 1: Schematic diagram of the influenza virion

The HA and NA spikes are inserted through the lipid membrane. Membrane protein M1 is the major structural protein and forms a layer on the inner surface of the lipid bilayer. The eight segments of RNA that comprise the genome are packaged as helical ribonucleoproteins containing NP and the polymerase proteins. Taken from Lamb, 1983.

history. Clinical isolates after a small number of passages are pleomorphic and often long filamentous particles are present (Horne et al., 1960). After multiple passage in eggs the virions are approximately spherical with a diameter of 100-120 nm when visualised by freeze-dryng and shadowing (Nermut and Frank, 1971).

A schematic diagram of a virion, taken from Lamb (1983) is shown in Figure 1. The outer surface of the virion is made up of 700-1,200 glycoprotein spikes anchored in a lipid bilayer. There are two types of projection: the more numerous haemagglutinin (HA) spikes are rod-shaped whilst the neuraminidase (NA) spikes are mushroom-shaped (Laver and Valentine, 1969; Wrigley et al., 1973; Wiley et al., 1983). There are about 4 to 6 times more HA than NA spikes and immunoelectron microscopy shows that the NA spikes occur in patches (Murti and Webster, 1986).

The lipid bilayer is derived from the host cell membrane during budding and amounts to over 20% of the mass of the virion (Kates et al., 1961). Underlying this is an electron-dense layer of M protein, approximately 6 nm thick (Apostolov and Flewett, 1969) accounting for 40% of the virion protein.

NP and the polymerase proteins, PB1, PB2 and PA are also internal. In combination with the virion RNA they form ribonucleoprotein (RNP) structures which are probably held together as helical nucleocapsids in virions by M (Lenard, 1978). Analysis of the RNPs liberated by disruption of virions shows that the RNA segments are separate and provide

evidence for specific association with M (Rees and Dimmock, 1981). RNPs appear as flexible rods and their lengths, 30-110 nm, has been correlated with the molecular weights of the genome segments (Compans et al., 1972). There are 500-940 A molecules of NP per virion (Compans and Choppin, 1975) and it is therefore calculated that each molecule interacts with approximately 20 nucleotides of RNA. It is thought that the RNA is exposed on the surface of the RNP complexes because it can be displaced by polyvinyl sulphate (Pons et al., 1969) and can be digested by ribonuclease without disrupting the RNP structure (Duesberg, 1969; Kingsbury and Webster, 1969; Pons et al., 1969; Murti et al., 1980). It has been estimated that there are 10 to 20 molecules of each of the polymerase proteins per virus particle (1.1 to 2.2% of the total protein)(Kawakami et al., 1981a).

There may also be other proteins in virions. Recent evidence suggests that a protein previously classed as a non-structural protein, M2, occurs in virions (see 3(e) below). Host proteins are almost completely excluded from the membrane during assembly (Holland and Kiely, 1970), but the presence of a membrane-bound host carboxypeptidase has been reported (Barten and Klenk, 1983).

The genome segments are numbered 1 to 8 in decreasing order of electrophoretic mobility on glyoxal gels (Desselberger and Palese, 1978) and gene assignment has been reviewed by Lamb (1983) and Lamb and Choppin (1983). Segments 1 to 6 code for single polypeptides whereas segments 7 and 8 each encode two polypeptides.

3. GENE PRODUCTS

(a) RNA Segments 1,2 and 3: the polymerase proteins PB2, PB1 and PA

The three largest RNA segments code for the polymerase proteins PB2, PB1 and PA respectively, designated on the basis of mobility during isoelectric focusing and electrophoresis (Horisberger, 1980; Ulmanen et al., 1981). The polymerase proteins are present as a complex composed of PB1, PB2 and PA in nucleocapsids and in infected cells (Braam et al., 1983; Detjen et al., 1987). The complex transcribes mRNA (Kawakami and Ishihama, 1983; Kato et al., 1983) and also probably synthesizes progeny viral RNA. The mechanism of transcription is considered in detail later. In vitro PB2 binds to the 5' cap of heterologous mRNA that primes transcription (Ulmanen et al., 1981, 1983; Blaas et al., 1982a,b; Penn et al., 1982; Braam-Markson, et al., 1985) and PB1 is responsible for the initiation and elongation reactions of mRNA synthesis (Ulmanen et al., 1983; Romanas and Hay, 1984). These studies confirm results obtained with temperature-sensitive mutants (Krug et al., 1975; Scholtissek and Bowles, 1975; Palese et al., 1977) which additionally indicate a role for PA in vRNA synthesis (reviewed by Mahy, 1983).

Comparison of the sequences for A/PR/8/34 and A/NT/60/68 (H3N2) strains shows 97, 96 and 96% homology for PB1, PB2 and PA respectively at the amino-acid level and the PB2 and PA genes are closely related to those of H1N1 strains (Murphy and Webster, 1985).

(b) RNA Segment 4: the haemagglutinin (HA)

HA is the major envelope glycoprotein accounting for approximately 4/5th of the surface spikes and 25 to 35% of the virion protein. It is the viral attachment protein and probably causes fusion of viral and endosomal membranes thereby mediating penetration of viral nucleocapsids into the cytoplasm. Additionally HA is the neutralisation antigen and exhibits marked antigenic variation which is responsible for the recurrent outbreaks of influenza virus infection. Thirteen subtypes of HA have been identified though there is antigenic variation within subtypes (Katz et al., 1985).

The three-dimensional structure of HA released by bromelain (BHA) from A/Aichi/68 (H3) has been determined by x-ray crystallography. BHA lacks the C-terminal hydrophobic region of HA2 and a schematic diagram of the HA monomer is shown in Figure 2. Sequence comparisons with H1, H2 and other H3 subtypes show conservation of structurally important residues suggesting a similar three-dimensional structure for all HAs (Wilson et al., 1981).

Each HA spike is composed of three HA1-HA2 monomers which associate non-covalently. HA1, the N-terminal portion (molecular weight approximately 45,000 to 50,000), carries the 3-5 neutralisation sites (Wiley et al., 1981; Caton et al., 1982). Residues 226 and 228 contribute to the viral attachment site and amino-acid changes at these residues alter the ability of virus to bind to receptors (Rogers and Paulson, 1983; Naeve et al., 1984; Rogers et al., 1985).

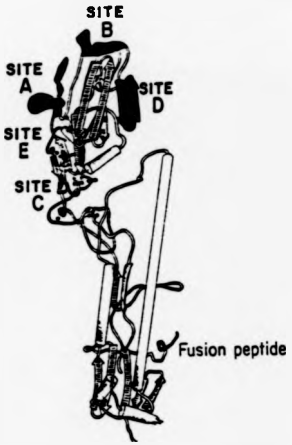


Figure 2: Sketch of the three-dimensional structure of the H3 haemagglutinin showing the positions of the antigenic sites

The shaded areas show the probable location of the antigenic sites. In the trimeric HA spike site D is buried and site E is often obscured by carbohydrate. Taken from Wilson et al. (1981) and Caton et al. (1982).

The intact HA2 has a molecular weight of 25,000 to 30,000 and contains two hydrophobic regions. The C-terminal hydrophobic region of 25-32 amino acids traverses the membrane, anchoring the HA. The N-terminal hydrophobic region, present in BHA, is the most highly conserved region of the HA and is thought to be responsible for penetration of virus into cells.

HA has been studied as a model for synthesis and transport of cellular proteins (see Garoff, 1985 for review; Doyle et al., 1985; Gething et al., 1986b). HA is synthesized as a monomeric subunit (Figure 3) on polysomes bound to the endoplasmic reticulum. The elongating chain is inserted into the vesicle of the endoplasmic reticulum by the N-terminal signal peptide and transferred to the lumen where initial glycosylation and folding of the polypeptide takes place. HA trimers form before the protein is transported to the Golgi apparatus (Gething et al., 1986b). Two proteolytic modifications are involved in maturation. Firstly, the signal peptide comprising 16 amino acids is removed. Later a trypsin-like enzyme and a carboxypeptidase cleave the molecule, remove an arginine residue, and sometimes other amino acids (Garten et al., 1982), yielding a monomer of HA1-HA2 covalently linked by a disulphide bond. The hydrophobic C-terminal sequence of HA2 anchors the HA in the membrane and 10 amino acids are exposed on the intracellular side of the membrane.

Proteolytic cleavage to HA1 and HA2 is dependent on virus strain, host-cell type and growth conditions (Lazarowitz et al., 1971, 1973a,b; Klenk et al., 1972; Kawaoka et al., 1984). It can be effected in vitro using trypsin-like enzymes (Appleyard and

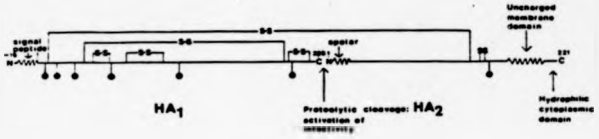


Figure 3: Schematic diagram of the H3 HA sequence.

The N-terminal sequence, which is cleaved off during co-translational membrane insertion is shown. The position of the C-terminal uncharged membrane anchoring domain is shown, together with the site of proteolytic cleavage which yields polypeptide chains HA1 and HA2 which is accompanied by activation of infectivity. The disulphide bonds are shown and carbohydrate attachment sites are illustrated by the line and circle. Adapted from Wilson et al. (1981) by Lamb (1983).

Maber, 1974; Lazarowitz et al., 1973a) and results in conformational (Skehel et al., 1982) and antigenic changes (Jackson and Nesterowicz, 1985) in the HA. Receptor binding is not dependent on cleavage (Lohmeyer and Klenk, 1979; McCauley et al., 1980) and virus with uncleaved HA attaches to cells, but is not internalised (Scholtissek, 1986). Proteolytic cleavage however is required for full infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975) and for in vitro membrane fusion activity (Huang et al., 1980a). Cleavage of the HA increases pathogenicity (Bosch et al., 1979; Rott, 1979; Kawaoka et al., 1984; Klenk et al., 1984) and facilitates spread of infection in the host (Rott et al., 1980; Bosch et al., 1981; Rott and Klenk, 1986). Administration of a protease inhibitor to infected mice resulted in a 100-fold reduction in virus yield, the HA of the progeny virus was uncleaved and the mice were able to clear the virus (Zhirnov et al., 1984). In mice influenza virus infection is potentiated by strains of Staphylococcus aureus that provide proteases which activate the HA (Tashiro et al., 1987a,b).

After co-translational core glycosylation in the endoplasmic reticulum (Compans, 1973b; Hay, 1974; Klenk et al., 1974; Elder et al., 1979; McCauley et al., 1980) further carbohydrate residues are added and trimmed during transport to the plasma membrane (Compans, 1973b; Rott and Klenk, 1977). In the mature H3 molecule there are four glycosylated sites on HA1 and one on HA2 (Ward and Dopheide, 1981; Wilson et al., 1981). The extent of glycosylation and the position of the carbohydrate residues influences the ability of HA to bind receptors (Daem et al., 1986) and its antigenicity. Rabbit and goat antisera raised against influenza virus react poorly with deglycosylated HA

(Alexander and Elder, 1984) and a mutation has been described that introduces a new glycosylation site resulting in the masking of an epitope (Skehel et al., 1984). Additionally the loss of a glycosylation site has been shown to facilitate cleavage of HA to HA1 and HA2 and thereby enhance virulence (Kawaoka et al., 1984).

(c) RNA Segment 5: nucleoprotein (NP)

NP (molecular weight 56,000) is one of the type specific antigens used to distinguish between A, B and C viruses, but some minor variations occur within the NP of type A viruses (Schild et al., 1979; van Wyke et al., 1980). It is the major nucleocapsid protein: there are estimated to be 500-940 molecules per virion (Compans and Choppin, 1975).

NP is arginine-rich and binds RNA in structural nucleocapsids (RNA-) and replicative nucleocapsids (RNA+). It was thought to be involved in RNA polymerisation but this appears unlikely as NP-free, RNA-protein complexes capable of RNA synthesis have been produced (Kawakami and Ishihama, 1983; Kato et al., 1985). Therefore, NP has no enzymic role in RNA synthesis and the observed inhibition of transcription in vitro by anti-NP antibodies (van Wyke et al., 1980) must be due to steric hindrance. However, NP may play a role in RNA replication by causing the switch from transcription of mRNA (incomplete transcripts of vRNA) to template cRNA by preventing termination of transcription at polyadenylation sites (Beaton and Krug, 1986). In the nuclei of infected cells free NP was found as well as NP associated with nucleocapsids and, by depletion, it was shown that it was the free NP that acted as antiterminator

(Beaton and Krug, 1986).

Temperature-sensitive NP mutants have been isolated that yield no infectious progeny at the non-permissive temperature (Scholtissek and Bowles, 1975) but which synthesize normal amounts of virion components suggesting that assembly is defective. Nucleocapsids interact with M (Rees and Dimmock, 1981) supporting the proposal that virion assembly involves such an interaction at the plasma membrane (Choppin et al., 1972; Compans and Choppin, 1975) as postulated for Sendai virus (Shimizu and Ishada, 1975).

NP is a phosphoprotein and exists in different phosphorylation states in the same cell (Privalsky and Penhoet, 1977, 1978; Almond and Felsenreich, 1982) and the extent of phosphorylation varies between cell types (Kistner et al., 1985). Phosphorylation occurs within ten minutes of synthesis and the proportion of NP which is phosphorylated increases as infection progresses (Almond and Felsenreich, 1982). The functional significance of this observation is not clear. Phosphorylation of NP in nucleocapsids increases transcriptase activity in vitro (Kamata and Watanabe, 1977) and in vivo it has been suggested that phosphorylation is associated with transport of NP to the nucleus (Almond and Felsenreich, 1982). However, using the same virus-cell combination Petri and Dimmock (1981) found no evidence to support this.

Further heterogeneity of intracellular NP results from proteolytic cleavage in some cell types. Late in infection a 53,000 molecular weight product accumulates but the full size protein (56,000 molecular weight) is preferentially packaged into

virions (Zhirnov and Bukrinskaya, 1981).

Five groups of NP genes have been defined by competitive RNA-RNA hybridisation analysis. Human and swine strains fall into one group, avian strains fall into two groups and equine strains make up the remaining two groups (Bean, 1984). NP may therefore contribute to the determination of species specificity (Tian et al., 1985).

(d) RNA Segment 6: the neuraminidase (NA)

NA is an integral membrane protein named for its receptor destroying activity which cleaves terminal n-acetyl neuraminic acid (sialic acid) residues from carbohydrate chains in glycoproteins (Gottschalk, 1957). The mushroom-shaped NA spikes (molecular weight approximately 220,000) compose approximately 1/5th of the envelope spikes. Each spike is a tetramer (Colman et al., 1983) and the constituent polypeptides (molecular weight approximately 56,000) are linked by disulphide bonds (Lazdins et al., 1972). There are nine subtypes of NA and the three-dimensional structure of the N2 subtype has been determined (Varghese et al., 1983).

Biosynthesis of the NA follows a similar pathway to HA. NA is co-translationally inserted into the membrane of the endoplasmic reticulum where the nascent polypeptide is core glycosylated and may assemble into tetramers (Breuning and Scholtissek, 1986). Further glycosylation occurs during transport to the plasma membrane (Compans, 1973b; Stanley et al., 1973; Hay, 1974; Klenk et al., 1974) but proteolytic cleavage is not involved in

maturation of NA. A single extended hydrophobic region (residues 7 to 35) acts as both signal sequence and anchors the protein by its N-terminus in membranes (Fields et al., 1981; Blok et al., 1982).

The role of the NA is unclear but its enzyme activity has been postulated to be required for fusion of cell and viral membranes (Huang et al., 1980b), release of progeny virus from infected cells (Palese and Schulman, 1974; Palese et al., 1974), production of sialic acid free virions (Klenk et al., 1970) with correctly cleaved HA (Schulman and Palese, 1977) and penetration of virus through the barrier of sialomucoproteins in the respiratory tract (Lamb and Choppin, 1983).

Whilst the HA is responsible for membrane-fusion, NA may play an accessory role (see Part 4 below). The most convincing evidence for NA contributing to membrane-fusion is the observation that fusion is prevented by inhibition of activity with anti-NA antibody (Huang et al., 1981) and restoration of fusion by addition of Vibrio cholerae neuraminidase (Huang et al., 1985). Tissue culture cells expressing only HA can be fused to each other (White et al., 1982) but this does not exclude a role for neuraminidase activity as animal cells contain endogenous neuraminidases (Schauer, 1983).

The suggestion that NA is responsible for release of progeny virus by freeing virus from sialic acid residues on host cells arises from studies with antibodies and temperature-sensitive mutants (Sato and Rott, 1966; Compans et al., 1969; Palese et al., 1974) but is probably incorrect. Virus particles are formed

and released when viral NA is inactive (Scholtissek and Bowles, 1975; Breuning and Scholtissek, 1986) or inactivated by monovalent antibody fragments (Becht et al., 1971). Antibodies probably prevent release by opposing the curvature of the cytoplasmic membrane during bud-formation (Dubois-Dalcq et al., 1984) but release of virus particles with inactive NA may involve endogenous neuraminidase (Schauer, 1983) and the argument remains unresolved.

NA may contribute to maturation of virus by depletion of sialic acid residues from virions (Klenk et al., 1970). A temperature-sensitive mutant defective in NA activity gave rise to clumps of virus at the non-permissive temperature (Palese et al., 1974). The progeny contained sialic acid residues not normally present in virions to which it was postulated the HA bound. Removal of sialic acid residues from HA is thought be necessary for proteolytic activation (Schulman and Palese, 1977) but there appears to be no strict correlation between NA activity and pathogenicity (Bosch et al., 1979). A reassortant with NA that is inactive above 40°C was as pathogenic by intra-muscular inoculation into chickens (body temperature 41°C) as the FPV parent virus (Breuning and Scholtissek, 1986).

Finally, the target cells for influenza in many animals form the epithelium of the respiratory tract. The tract is lined with sialic acid-containing glycoproteins to which the HA appears to bind, reducing infectivity (Shen and Ginsberg, 1968). The probability of a given virion reaching a cell would be increased if the NA released virus attached to free glycoproteins and it may be that the function of viral NA is to permit entry at the

site of natural infection (Breuning and Scholtissek, 1986). Related to this, NA can modify gangliosides raising the possibility that NA alters a primary receptor to a form recognised by HA (Slepushkin et al., 1985).

(e) RNA segment 7: Membrane protein M1, and M2 and M3

Genome segment 7 gives rise to three mRNAs (Lamb et al., 1981) but translation products have been identified for only two. The relationship between the genome segment, mRNAs and polypeptides is shown in Figure 4.

M1 (molecular weight 28,000) is the second type-specific antigen though minor antigenic differences are detectable (LeCorate and Oxford, 1981; van Wyke et al., 1984). In common with NP it is a phosphoprotein (Gregoriades et al., 1984) although phosphorylation is not obligatory for replication (Kistner et al., 1985) and M1 has been implicated in host-range restriction (Klimov et al., 1983; Tian et al., 1985; Buckler-White et al., 1986). M1 is encoded by 75% of an mRNA that is colinear to the genome segment (Lamb, 1983) which is translated on free ribosomes (Compans, 1973a; Hay, 1974). Since it lacks a signal sequence, M1 is transported to the cell surface by a membrane-independent pathway.

M1 is thought to play an important structural role and this is supported by observations that virus particles with reduced quantities of M1 are more fragile (Kendal et al., 1977; Bukrinskaya et al., 1981). Furthermore, M1 is the most abundant structural protein (Skehel and Schild, 1971) and, at an estimated

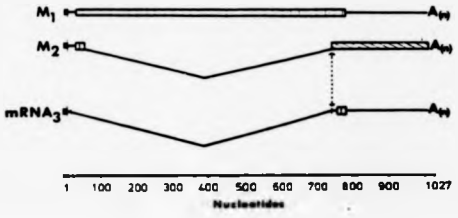


Figure 4: Diagram showing the relationship of genome segment 7 to the three mRNAs specified.

The shaded boxes represent the coding regions and indicate the reading frame i.e. M1 and the putative M3 peptide are in the same reading frame distinct from that of M2. The thin lines are non-coding regions and the V-shaped regions are removed by splicing. The filled boxes mark the position of host-derived primer sequences (see Figure 6). Adapted by Kingsbury (1985) from Lamb (1983).

3,000 to 4,000 molecules per virion, is the only protein present in sufficient quantity to form the electron-dense layer that underlies the lipid bilayer in virions (Apostolov and Flewett, 1969; Compans and Dimmock, 1969; Bachi et al., 1969; Compans et al., 1972; Schulze, 1972). Proteolytic digestion of intact virions removes the glycoprotein spikes but M1 remained intact (Compans et al., 1970) consistent with location of M1 beneath the lipid. M1 is external to nucleocapsids (Stanley and Haslam, 1971; Rifkin et al., 1972; Lenard et al., 1974) and following infection of cells subviral particles containing M1 and NP are found associated with nuclei of cells (Hudson et al., 1978; Bukrinskaya et al., 1980; Bukrinskaya et al., 1982).

Related to its structural role, M1 is implicated in viral assembly (Choppin et al., 1972; Compans and Choppin, 1975; Dubois-Dalcq et al., 1984) in a way analogous to that suggested for the corresponding M protein in the assembly of rhabdoviruses (Shimizu and Ishida, 1975) where, in the case of VSV, M protein causes coiling of nucleocapsids (Heggeness et al., 1980). M1 interacts with lipid and may traverse the bilayer (Bucher et al., 1980; Gregoriades, 1980; Gregoriades and Frangione, 1981; Karadaghi et al., 1984; Tversidov et al., 1984), possibly by virtue of a hydrophobic region in the middle of the molecule, and is associated with the plasma membrane within minutes of synthesis (Lenard, 1978). M1 also binds to viral RNPs (Reginster and Nermut, 1976; Rees and Dimmock, 1981) but only after association with the plasma membrane (Hay and Skehel, 1974). Assembly appears to be determined by the ability of M1 to recognise glycoproteins at the cell surface. In some abortive infections and when defective M1 is produced, HA and NA are found

in the plasma membrane but neither M1 nor NP accumulate there (Lohmeyer et al., 1978; Maeno et al., 1981; Bukrinskaya et al., 1981). At the non-permissive temperature the nucleocapsids of a mutant with a temperature-sensitive lesion in M1 accumulated in nuclei (Enami et al., 1985) but the possible contribution of other mutations not associated with the ts phenotype were not considered. M1 increases the incorporation of NA into liposomes providing evidence for interaction of M1 and glycoproteins (Davies and Bucher, 1981).

In addition to its structural role, M1 may be involved in uncoating and the regulation of transcription. Amantadine and an analogue rimantadine are licensed anti-influenza drugs that inhibit virus replication in cell culture. At concentrations greater than 0.1 mM amantadine, like other lysosomotropic amines, elevates the endosomal pH preventing membrane fusion by the HA and mutants raised in the presence of high concentrations contain mutations in the HA (Daniels et al., 1985). At concentrations of 0.1-5 μM amantadine exerts strain-specific inhibition of initiation of infection or virus assembly (Appleyard, 1977). Sensitivity to low concentrations of amantadine has been mapped to the M2 sequence (Hay et al., 1985) as described below, but there is evidence that complete uncoating involves loss of M1 protein from sub-viral particles liberated into the cytoplasm from endosomes (Bukrinskaya et al., 1980; Bukrinskaya et al., 1982).

M1 inhibits transcription in vitro directed by nucleocapsids derived from virus by detergent treatment (Zvonarjev and Ghendon, 1980; Mikhejeva and Ghendon, 1983) and the level of transcriptase

activity of M1-free nucleocapsids is higher than that of M1-bound nucleocapsids (Kato et al., 1985). Inhibition of transcription by the corresponding M protein has also been described for VSV (Carrol and Wagner, 1979; Arnheiter et al., 1985; Pal et al., 1985; Ye et al., 1985) and a positive strand RNA virus, Banzi virus (Hommell-Berrey and Schloemer, 1986). Influenza virus transcription can be inhibited by M1 protein from different serotypes and M protein from VSV (Mikhejeva and Ghendon, 1983) lending weight to the suggestion that M proteins from different viruses recognise similar domains in other viral proteins, as proposed to explain pseudotype formation (Zavada, 1982). Coat proteins which have both structural roles and regulate activity of nucleic acid-replicating enzymes have been identified in alfalfa mosaic virus (Mohier et al., 1974), adenovirus (Korn and Horwitz, 1986) and brome mosaic virus (Horikoshi et al., 1987).

The other two segment 7 mRNAs are derived from M1 mRNA by splicing. Nucleotide sequences (Winter and Fields, 1980; Allen et al., 1980; Lamb and Lai, 1980; McCauley et al., 1982; Ortin et al., 1983) predict that M2 is a polypeptide of 97 amino acids and molecular weight of 11,000 but it migrates on gels with a molecular weight of 15,000 (Lamb and Choppin, 1981). The nine N-terminal amino acids are the same as for M1 but as a consequence of splicing the remainder of M2 mRNA is translated in the +1 reading frame (Lamb et al., 1981). Residues 25-43 are hydrophobic and this region is thought to traverse the membrane with the 54 C-terminal amino acids located on the cytoplasmic side. In support of these predictions it has been shown that M2 is an integral membrane protein with 18 to 23 N-terminal residues exposed on the cell surface (Lamb et al., 1985; Zebedee et al.,

1985). M2 has been classified as a non-structural protein since attempts to detect the protein in purified virus failed (Lamb and Choppin, 1981; Lamb et al., 1985). However, R.A. Lamb and co-workers have since immunoprecipitated M2 from purified WSN virus grown in eggs or MDCK cells (R.A. Lamb, personal communication). M2 is under-represented in virions: in WSN infected cells the ratio of HA:M2 is 1.5:1 (Lamb et al., 1985) but there are estimated to be only 20-60 molecules of M2 per virion. It is possible that the M2 detected is present in contaminating vesicles and not virus particles.

No function has yet been attributed to M2 but its expression in large quantities at the cell surface suggests a role in assembly (Lamb et al., 1985). Resistance to low concentrations of amantadine was mapped by complementation analysis to RNA segment 7 (Lubeck et al., 1978; Hay et al., 1979). Later, Hay et al. (1985) showed the primary site of action of amantadine to be M2 since amantadine-resistant mutants had amino acid substitutions within the hydrophobic domain (residues 27,30,31 and 34). Since low concentrations of amantadine prevent initiation of infection, a role for M2 is implied, consistent with M2 being a virion component (Hay et al., 1985).

The third transcript arising from RNA segment 7 is also a spliced mRNA and is found in infected cells (Lamb et al., 1981; Inglis and Brown, 1981). The nucleotide sequence predicts a nine amino acid peptide co-terminal with the carboxy end of M1. The presence of this peptide either in infected cells or virions remains to be demonstrated.

RNA Segment 8: the non-structural proteins NS1 and NS2

Like RNA segment 7, segment 8 is transcribed into a colinear transcript which codes for a protein, NS1 and is also spliced, in this case to produce one other mRNA, that for NS2 (Figure 5; Lamb, 1983; Lamb and Lai, 1984; Smith and Inglis, 1985). These two proteins, found only in infected cells, have their nine N-terminal residues in common but the remainder of NS2 is translated in the +1 reading frame (Lamb and Lai, 1980). The functions of these proteins remain unclear. One host-range mutant has been identified where segment 8 was aberrant (Maassab and DeBorde, 1983).

NS1 is a phosphoprotein (predicted molecular weight of 26,000, apparent molecular weight 23,000), has been implicated in viral RNA replication (Almond et al., 1977; Masno et al., 1979; Wolstenholme et al., 1980), inhibition of the processing of rRNA precursors and to a lesser extent host protein synthesis (Stephenson and Dimmock, 1974; Krug and Soiero, 1975) and regulation of virus-specific polypeptide synthesis (Inglis and Mahy, 1979; Wolstenholme et al., 1980). It is a phosphoprotein but like M1 phosphorylation is not obligatory for successful replication (Kistner et al., 1985). Large amounts are synthesized early in infection (Dimmock, 1969; Lazarowitz et al., 1971; Pons, 1972; Compans, 1973a; Krug and Etkind, 1973; Krug and Soiero, 1975) and it is found associated with polysomes and nucleoli (Lazarowitz et al., 1971; Skehel, 1972; Meier-Ewert and Compans, 1974). Late in infection with some strains cytoplasmic inclusion bodies composed of NS1 and cellular RNA form (Morongiello and Dales, 1977; Shaw and Compans, 1978; Yoshida et al., 1981) but

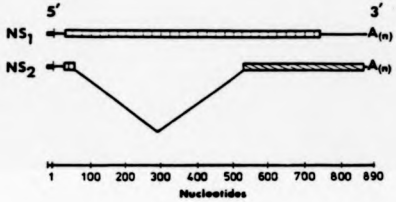


Figure 5: Diagram showing the relationship of genome segment 8 to the NS1 and NS2 mRNAs.

Symbols as used in Fig. 4.

this is probably simply a consequence of the basic nature of some NS1 proteins as the NS1 of FPV is acidic in infected cells and does not form inclusion bodies (Petri et al., 1982).

A polypeptide of 132 amino acids is predicted from the nucleotide sequence of NS2 (molecular weight 14,000, apparent molecular weight 11,000). No function has yet been ascribed to NS2 which is synthesized late in infection. Conflicting reports show accumulation of NS2 in the cytoplasm (Skehel, 1972; Follett et al., 1974; Minor and Dimmock, 1975; Lamb et al., 1978; Lamb and Choppin, 1979; Mahy et al., 1980; Briedis et al., 1981) and in the nucleus (Greenspan et al., 1985) and the basis for this difference remains to be resolved.

4. HA-MEDIATED MEMBRANE-FUSION

Isolated HA can cause membrane-fusion (Sato et al., 1983; Wharton et al., 1986) but fusion is most efficient when HA is anchored in a membrane (White et al., 1982). Thus, virus particles, liposomes incorporating HA or cells expressing HA at the plasma membrane can be fused with target membranes of liposomes, cells or red blood cells (RBCs). Virus particles can cause lysis of RBCs (haemolysis) which provides one method of assaying fusion.

HA-induced membrane-fusion requires a trigger, for example reduction of pH to an optimal value, which induces a change in conformation in the HA. The optimal pH values for fusion, conformational change and haemolysis lie in the range pH 5 to 6.4 (Daniels et al., 1985) and vary between different strains and

mutants. For HA from a given source the absolute value for the optimum pH for each of these three parameters may be different but are found to co-vary (Daniels et al., 1985). For example, mutation in the HA resulting in elevation of the pH at which conformational rearrangements is triggered will raise the optima for fusion and haemolysis.

(a) The role of HA in membrane-fusion

Infectivity is dependent on proteolytic cleavage and carboxypeptidase trimming of HA0 creating the amino-terminus of HA2. The terminal decapeptide is conserved, other than at position 2, in all influenza A viruses and is homologous with the amino-terminal sequence of the fusion (F) protein of the paramyxovirus Sendai virus (Skehel and Waterfield, 1975; Scheid and Choppin, 1977). The F protein, which is also activated by proteolytic cleavage, mediates fusion suggesting a role for HA in fusion.

Analysis of the three-dimensional structure of the neutral form of the HA trimer shows that the hydrophobic N-terminus of HA2 is buried in a hydrophobic cleft at the interfaces between the subunits and approximately 10 nm from the viral attachment site at the tip of the molecule. The change in conformation of the HA is postulated to involve partial dissociation of the HA exposing the ten hydrophobic residues. The low pH form is more hydrophobic than HA at neutral pH. On lowering the pH, BHA, which lacks the hydrophobic sequence near the carboxy-terminus of HA2, forms aggregates and acquires an affinity for lipids and detergents (Skehel et al., 1982; Doms et al., 1985).

The conformational change induced by low pH is irreversible and is detectable by a number of means. By electron microscopy the low pH-forms of intact and bromelain-released HA (BHA) appear elongated (Ruigrok et al., 1986a). Electron spin resonance spectra of spin-labelled HA (Kida et al., 1985) and circular dichroism spectra (Skehel et al., 1982) alter as the pH is lowered from neutral to the optimum for fusion. After low pH treatment the HA also becomes susceptible to protease digestion by trypsin (Skehel et al., 1982) and proteinase K (Doms et al., 1985; Gething et al., 1986a) and the disulphide bond linking HA1 and HA2 becomes sensitive to reduction with dithiothreitol (DTT) (Graves et al., 1983). These structural changes are limited and do not constitute denaturation of the HA, however (Skehel et al., 1982; Daniels et al., 1983; Nesterowicz et al., 1985). The low pH-form is still able to bind to glycophorin A, the major receptor for influenza virus on RBCs, (Jackson and Nesterowicz, 1985) and cause haemagglutination (Yewdell et al., 1983; Kida et al., 1985) indicating that the viral attachment site remains functional. Antigenic mapping of H1 and H3 subtypes with monoclonal antibodies has revealed maintenance of the structural integrity of some antigenic sites e.g. site A in H3 (Figure 2), alteration of others, for example sites A,C and D in the H3 molecule, and the appearance of a new determinant unique to the low pH-form (Yewdell et al., 1983; Webster et al., 1983; Jackson and Nesterowicz, 1985; Nesterowicz et al., 1985). Direct evidence that HA trimers do not dissociate to monomers after acidification was provided by Doms and Helenius (1986). Since BHA dissociated under similar conditions this suggests that the carboxy termini of HA2 maintain the trimeric structure.

Genetic analysis has led to an understanding of the amino acid residues that are important in fusion and elucidation of the mechanism. Variant viruses have been isolated and mutants produced which induce fusion at higher pH than the parent (Rott et al., 1984; Daniels et al., 1985; Doms et al., 1986). Interpretation of the sequence differences associated with this phenotype with regard to the three-dimensional structure of HA at neutral pH suggests that elevation of the pH of fusion is the result of substitutions that disrupt interactions at the interfaces of subunits which reduce the stability of the trimer (Rott et al., 1984; Daniels et al., 1985) or destabilise the conformation that holds the amino-terminal peptide in the hydrophobic crevice at pH 7 (Daniels et al., 1985; Doms et al., 1986).

By introducing single amino acid changes into the N-terminal decapeptide of HA2 by site-directed mutagenesis Gething et al. (1986a) have identified some of the stages involved in HA-induced membrane-fusion. All the mutant HAs produced underwent conformational rearrangements at low pH resulting in the HAs becoming sensitive to protease digestion and able to bind lipids and liposomes. However, fusion ability varied in three ways: substitution of glutamic acid (GLU) at the N-terminus with glycine (GLY) prevented fusion; GLU in place of GLY at position 4 raised the threshold pH and decreased fusion efficiency, whilst a mutant with GLY instead of GLU at position 11 was able to fuse RBCs to cells expressing HA, but not induce cell-cell fusion. Consequently these authors suggest that fusion involves at least

three stages: exposure of the fusion peptide by conformational rearrangement of the HA; destabilisation of the target lipid bilayer by interaction of the fusion peptide and possibly other regions of the HA with the bilayer, and finally fusion itself as a consequence of destabilisation. Since the HA can bind to liposomes but not cause fusion this suggests that destabilisation may be dependent on the conformation that the fusion peptide assumes in the target membrane (Gething et al., 1986a) but absolute conservation of the amino acid sequence is not required (Daniels et al., 1985). Fusion of RBCs to cells expressing HA but not induction of polykaryon formation was interpreted as inability to cause lipid bilayer destabilisation over sufficiently large areas (Gething et al., 1986a). However, the difference may be due to differences in the composition of the cell envelopes. Fusion of virus to liposomes (Haywood and Boyer, 1985) is affected by the lipid composition and efficiency of fusion of liposomes to HA-expressing cells is dependent on composition of both the liposome target membrane and the cell type (van Meer et al., 1985).

In the infected cell pH is thought to trigger HA-induced membrane-fusion in endosomes, resulting in introduction of viral genetic material into the cytoplasm (review: Patterson and Oxford, 1986) but exposure to mildly acidic pH is not the only means of activating HA-mediated membrane-fusion. Virus has been fused with liposomes at neutral pH and elevated temperature for example 62°C for X-31 (Ruigrok et al., 1986b). Analysis of mutants of this virus showed that the higher the optimum pH for fusion at 37°C then the lower the temperature required to trigger fusion at neutral pH (Ruigrok et al., 1986b; Wharton et al.,

1986). At the temperature of fusion the BHAs underwent an irreversible conformational change as judged by protease sensitivity, CD and electron microscopy. However, the rearrangement was more pronounced than that induced by pH and resulted in partial denaturation of the molecule (Ruigrok et al., 1986b).

Influenza virus will also fuse to liposomes at pH 7.5 and 37°C if the liposomes carry a net negative charge as a result of incorporation of suitable lipids or the ganglioside GD1a (Haywood and Boyer, 1985). As well as imparting a negative charge to the liposomes, GD1a may also be a receptor for HA (Slepushkin et al., 1985). Since the time-course of fusion of influenza virus membranes with these liposomes parallels the time-course for binding and entry of influenza virus into cells, it has been postulated that virus may fuse with cells at neutral pH and gain entry via the plasma membrane (Haywood and Boyer, 1986).

(b) The role of NA in membrane-fusion

Whilst the HA is responsible for membrane-fusion, the studies of Huang and co-workers strongly implicate a role for neuraminidase activity in the process. An absolute requirement for the NA in fusion of liposomes containing influenza glycoproteins with cell membranes has been demonstrated (Huang et al., 1980a,b) and cell fusion and haemolysis are not induced by virus particles when NA activity is inhibited by antibody (Huang et al., 1981). The strongest evidence supporting a role for NA is that fusion of erythrocytes and haemolysis are induced when virus with the NA inactivated by antibody is supplemented with Vibrio cholerae

neuramindase (Huang et al., 1985) but the relevance of erythrocyte-virus fusion to penetration of infectable cells remains unclear. Evidence against the involvement of NA in membrane-fusion is that influenza B virus in which the NA activity has been heat-inactivated causes haemolysis (Shibata et al., 1982) and CV-1 cells expressing cloned HA fuse when exposed to low pH (White et al., 1982). Additionally, membrane-fusion is involved in penetration of virus into cells (see Part 5, below), but anti-NA antibodies do not neutralise infectivity (Seto and Rott, 1966). However, since some residual NA activity remained after heat-treatment (Shibata et al., 1982) and animal cells contain endogenous neuraminidases (Schauer, 1983) these studies do not rule out a requirement for neuraminidase activity in membrane-fusion.

5. ENTRY OF ENVELOPED VIRUSES INTO CELLS BY PINOCYTOSIS

(a) Model for entry of enveloped viruses

Enveloped viruses introduce their genetic material into cells by fusion of the viral membrane with a cellular membrane. In the case of some viruses, for example Sendai virus, pH-independent fusion occurs at the cell surface (Poste and Pasternak, 1978). In common with some other enveloped viruses influenza virus is taken up into cytoplasmic vesicles by endocytosis and fuses with the membrane of endocytic vesicles under acidic conditions (see Lenard and Miller, 1983; Marsh, 1983; Patterson and Oxford, 1986 for reviews). The entry pathway for Semliki Forest virus (SFV) into BHK cells has been most extensively studied and influenza virus,

VSV and rabies virus appear to follow a similar route. Therefore, the entry pathway for SFV will be described first followed by the evidence that influenza virus gains entry in a similar fashion.

SFV binds to an unidentified receptor on the microvilli of BHK-21 cells and migrates to clathrin-coated pits where it is taken up into cytoplasmic vacuoles (Helenius et al., 1980). These cytoplasmic or pinocytic vacuoles are initially lined with clathrin (Peterson and van Deurs, 1983) but this is recycled back to the plasma membrane. SFV can be bound to cells but prevented from entering by incubation at 4°C. On warming to 37°C the components of bound virus are found in coated vesicles in 15 sec and after 1 minute reach endosomes (Helenius et al., 1980). Other workers find that SFV can fuse at 0°C (J.Davey, personal communication). Endosomes are independent organelles consisting of a large vacuole with approximately six radial tubular extensions and are located either in the peripheral cytoplasm or the perinuclear region (Marsh et al., 1986). After 20 minutes the viral components are found in lysosomes, originally thought to be the site of intracellular penetration because agents that raise the lysosomal pH, and thereby prevent fusion, inhibit infection. However these agents, for example NH_4Cl, also raise the pH in endosomes where the time-course and temperature dependency of infection suggest fusion occurs. Within 5 to 7 minutes of leaving the cell surface and before viral components reach lysosomes, uncoated SFV RNA is found in the cytoplasm and NH_4Cl does not inhibit infection if added after 10 minutes (Helenius et al., 1980). Additionally, SFV uncoats and initiates infection at 20°C (Marsh et al., 1983) but transport from endosomes to lysosomes does not occur at this temperature (Dunn et al., 1980; Marsh et

al., 1983; Yoshimura and Ohnishi, 1984).

(b) Entry of influenza virus

HA attaches to sialic acid-containing residues which are the cell receptor sites for influenza viruses. The ability to recognise specific sialyloligosaccharides varies between different strains and within the same subtype (Paulson et al., 1979; Carrol et al., 1981; Rogers and Paulson, 1983; Underwood et al., 1987; Daniels et al., 1987). H3 subtypes recognise either one or both of the sequences NeuAcα2-6 Gal (α2-6 linkage of sialic acid, NeuAc) or NeuAcα2-3 Gal (α2-3 linkage)(Rogers and Paulson, 1983) and isolates that recognise the α2-6 linkage can vary in the ability to recognise other configurations (Rogers et al., 1985). The specificity of the HA is determined by the amino acid residues in and around the pocket at the distal tip of the HA that is the viral attachment site (Wilson et al., 1981; Rogers et al., 1983, 1985). The expression of sialic acid residues on host cells is species- and tissue-specific, and therefore differential recognition is a determinant of species tissue tropism (Naeve et al., 1984; Rogers et al., 1983; Higa et al., 1985) and the host cell can select a sub-population of one specificity (reviewed by Schild et al., 1984; Rogers et al., 1985). Like SFV, influenza virus preferentially binds to microvilli (Bachi et al., 1969; Helenius et al., 1980; Matlin et al., 1981) but in polarised cells infection can occur via both the apical and basolateral surfaces with similar efficiency (Fuller et al., 1984).

Having bound to the cell receptor unit (CRU), influenza virus-CRU complexes undergo redistribution (Patterson and Oxford, 1986)

which may facilitate entry. Virus is internalised providing that the HA is in the proteolytically cleaved, active form (Scholtissek, 1986). Influenza virus has been observed in coated pits but the majority is probably taken up at uncoated pits (Patterson et al., 1979) and penetration can occur at low temperatures i.e. at 0-4°C (reviewed by Dimmock, 1982; Results and Discussion Section IV). In conjunction with data showing penetration in the presence of metabolic and cytoskeletal inhibitors, this suggests an energy independent uptake mechanism for influenza virus (Patterson et al., 1979). In the low pH environment of the endosome fusion activity of the HA is triggered and the mechanism of fusion has been described (Part 5, General Introduction). Agents that raise the endosomal pH e.g. NH_4Cl and amantadine, inhibit infection but do not prevent replication if added 10 minutes after inoculation at 37°C or 1 hour at 20°C, the time taken for the environment around the virus to become acidified to approximately pH 5 (Yoshimura and Ohnishi, 1984). Additionally, a monoclonal anti-HA antibody specific for conformationally altered HA binds to internalised virus but not to virus adsorbed to the cell surface (Bachi et al., 1985), consistent with the HA undergoing the pH-induced conformational change which is thought to fuse the membranes of the virus and endosome and liberate virion RNA-containing cores into the cytoplasm. This antibody also reacts with virus bound to plastic and is therefore a marker of conformational change, not necessarily that induced by mildly acidic pH. In this way fusion is accompanied by uncoating, but there is evidence that this represents only partial uncoating as described in the next section. Since both RNA transcription and replication take place in the nucleus (Shapiro et al., 1987), these cores must be

transported to the nucleus. Little is known about the mechanism by which this transport occurs but it is assumed to be ordered since 50% of virion RNA internalised reaches the nucleus after 15 minutes (Stephenson and Dimmock, 1975). A sequence of the NP gene that controls nuclear accumulation of this protein in Xenopus oocytes has been identified (Davey et al., 1985a) which may be important.

6. UNCOATING OF INFLUENZA VIRUS

The process of uncoating of influenza virus remains ill-defined. Experiments to understand the process have relied on limiting the early events by carrying out inoculation at 4°C or using inhibitors.

At 4°C influenza virus penetrates into cells and the virion RNA enters the nucleus, but no virus-specific synthesis occurs (Stephenson and Dimmock, 1975). When the temperature is raised to 37°C primary transcription begins and virion RNA emerges from the nucleus. The migration of virion RNA from the nucleus is puzzling considering that the nucleus is the site of transcription. Whilst the lipid component remains in the cytoplasm, half of the NP and M associates with the nucleus and unquantified amounts of HA1 and HA2 are found in both the cytoplasmic and nuclear fractions. Analysis of the sedimentation behaviour of the viral components in nuclei showed that they were ribonucleoprotein particles (RNP) with similar composition to RNP obtained by disruption of virions with NP40 (Hudson et al., 1978).

The data of Bukrinskaya et al. (1980, 1982) suggest that a secondary stage of uncoating may be involved subsequent to loss of the lipid, and presumably the glycoproteins, within endosomes. These workers compared the relative quantities of viral components in cytoplasm, nuclear associated cytoplasm (NAC) and nuclei of cells incubated in the absence and presence of rimantadine. Rimantadine and amantadine, derivatives of adamantane, at low concentrations allow attachment and penetration but not primary transcription of sensitive strains (Hay et al., 1985). In the absence of rimantadine the NAC contained both M1-containing nucleocapsids (M1-RNP) and M1-free nucleocapsids (RNP) and the nuclei contained RNP. In addition M1 was associated with the nuclear envelope (Bukrinskaya et al., 1982). In the presence of the drug, M1-RNP were found in both the NAC and nuclei. These results are consistent with a scheme involving loss of the lipid and glycoproteins within endosomes, primary uncoating, followed by a second stage involving loss of M1 protein to produce RNP. In similar experiments Kozeletskaya et al. (1984) reported attachment, penetration and lack of primary transcription in the presence of rimantadine, but found no correlation with nucleocapsid composition.

Experiments with photosensitive virus also indicate that a stage subsequent to penetration is interrupted by amantadine and rimantadine. Photosensitive virus is prepared by growth in the presence of neutral red and remains sensitive to light until early in infection. In the presence of amantadine Kato and Eggers (1969) demonstrated that virus attached to and penetrated cells, but remained photosensitive. Similarly, Bubovich et al. (1985) found that virus remained photosensitive in the presence of

rimantadine and also showed that virion RNA was transported to the nucleus.

Taken together these results strongly suggest that complete uncoating involves more than loss of the lipid and glycoproteins. The second stage may be loss of M1 from nucleocapsids (Bukrinskaya et al., 1980, 1982) or some other event leading to primary transcription. Loss of M1 is an attractive proposition because this protein inhibits transcriptase activity in vitro (Zvonarjev and Ghendon, 1980; Mikhejeva and Ghendon, 1983). It is intriguing that amantadine resistant mutants contain amino acid substitutions in the membrane spanning region of M2 (Hay et al., 1985) which is either absent from virions or present in very low amounts (Part 3(e) above).

After inoculation onto cells the virion RNA becomes sensitive to RNase digestion and this can be used as a measure of uncoating (Koff and Knight, 1979). In the presence of rimantadine, sensitivity of virion RNA to RNase did not increase significantly (Koff and Knight, 1979) but, since these workers did not demonstrate penetration, this could be the result of a failure of primary or secondary uncoating.

The end of the initial stages of infection namely attachment, penetration and uncoating is marked by the presence of virion RNA-containing nucleocapsids in the nucleus in a form capable of acting as template for the active transcriptase complex.

7. TRANSCRIPTION AND RNA REPLICATION

Transcription and RNA replication are executed by viral gene products but there is an obligatory requirement for a functional host-cell nucleus. Viral replication does not occur in enucleate cells (Follett et al., 1974; Kelly et al., 1974), cells irradiated with UV light prior to infection, nor in the presence of actinomycin D (Barry et al., 1962). Specific inhibition by α-amanitin of cellular RNA polymerase II, which is responsible for DNA-dependent mRNA transcription, also inhibits influenza virus transcription (Rott and Scholtissek, 1970; Lamb and Choppin, 1977; Spooner and Barry, 1977). Depletion of mRNA in the nucleus by these means affects replication because the viral polymerase enzyme, present in the virion, lacks the ability to cap and methylate RNA and relies on the host cell for these functions. Other inhibitors of nuclear function also prevent replication (Minor and Dimmock, 1977). Production of viral mRNAs in the infected cell that are capped and methylated (Plotch et al., 1978) is achieved by pilfering ready-made cap structures from cellular mRNAs that act as primers for viral transcription. The mechanism of "trans-capping" has been elucidated largely from the study of transcription directed by purified virions in vitro and is considered next.

(a) Transcription in vitro

Polymerase activity of virus is activated by disrupting the membrane of virions, for example by detergent treatment, and is present in nucleocapsids purified from virions. Initiation of transcription occurs efficiently only in the presence of suitable

mRNA and dinucleotide primers and cap analogues.

To act as primers of transcription mRNAs must have a 5' cap methylated at position 7, be 2' O-methylated at the penultimate base (Bouloy et al., 1980) and have an adenine (A) residue 11-14 nucleotides from the 5' end (Plotch et al., 1981). The capped RNA is bound by PB2 (see Figure 6 for mechanism)(Ulmanen et al., 1981, 1983; Blaas et al., 1982a,b; Penn et al., 1982; Braam-Markson et al., 1985) and cleaved after the A residue by an unidentified protein. This produces a capped oligonucleotide with a hydroxyl group at the 3' terminus that can act as a primer for transcription. The A residue base-pairs with the uridine (U) residue at the 3' end of the virion RNA and the first nucleotide added by PB1 of the viral enzyme complex is a guanosine (G) residue complementary to the penultimate base of each genome segment. Thus, mRNA synthesis is initiated and repeated addition of complementary nucleotides by PB1 leads to elongation of the mRNA (Braam et al., 1983; Romanos and Hay, 1983; Ulmanen et al., 1983). The cap remains bound to the complex whilst the first 10-20 nucleotides are added (Braam et al., 1983).

Whilst PB1 appears to be solely responsible for initiation and elongation, the three polymerase proteins remain associated as a complex moving down the template as the transcript is synthesized (Braam et al., 1983). The resulting transcripts contain a capped, heterologous sequence covalently linked to the virus-specific sequence. Transcription terminates at an oligo U tract 5-7 residues long between 15 and 22 bases from the 5' end of the vRNA (Robertson et al., 1981) which constitutes the polyadenylation site. The mechanism of polyadenylation is not understood but may

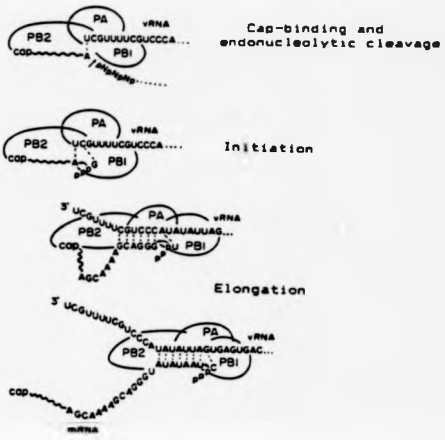


Figure 61 Model of the functions, interactions, and movements of the three P proteins in the initial stages of influenza virus mRNA synthesis.

The RNA sequences shown are those of the NP (genome segment 5). PB2 binds to the 5' terminal cap of heterologous RNA which is then cleaved at a purine residue 10-13 nucleotides from the cap (sequencing of mRNAs produced *in vivo* suggests that preferential cleavage occurs after the sequence (G)CA). PB1 catalyses the addition of virus-specific complementary nucleotides in the initiation and elongation reactions. The three polymerase proteins move together along the template, the cap being released from PB2 after 10-20 nucleotides have been added to the primer. Adapted from Braam *et al.* (1983) by Kingsbury (1985).

be the result of repetitive copying of the oligo U tract or be template-independent (Krug, 1983).

Synthetic oligonucleotides and cap analogues also stimulate transcriptase activity. Of the synthetic oligonucleotides, ApG (3'-5'), XpApG and ApGpC are the best primers (Plotch and Krug, 1977; Kawakami et al., 1981; Stridh et al., 1981b) probably because they base-pair with the conserved sequence at the termini of vRNA segments. However, these are at least 1,000 times less efficient as primers than mRNA on a molar basis (Bouloy et al., 1978). ApG forms the 5' end of transcripts (Figure 7; Plotch and Krug, 1977) which are polyadenylated and are translated in some cell-free systems (Krug, 1983). Heptanucleotides complementary to the vRNA do not act as efficiently as primers as the oligonucleotides mentioned above, perhaps because the 3' termini of the heptanucleotides are too far from the active site of the RNA polymerase when annealled to the vRNA (Kawakami et al., 1981b).

Cap analogues, for example m^7GpppAm, stimulate transcription, but are not incorporated into transcripts and the effect is additive with ApG (Penn and Mahy, 1984; Kawakami et al., 1985). Such stimulation therefore appears to be allosteric. No analysis of the structure of the transcripts produced in the presence of cap analogues has been reported but presumably these are similar to those synthesized in the presence of ApG and have an uncapped 5' end complementary to the 3' terminus of vRNA. m^7GMP stimulates transcription and is incorporated into transcripts. Since the effect is not additive with ApG this suggests that these molecules prime by a similar mechanism (Penn and Mahy, 1984).

(b) RNA synthesis *in vivo*

Influenza virus mRNAs produced *in vitro* and *in vivo* have a number of similarities, implying a common mechanism of synthesis. Both contain a host-donated sequence at the 5' terminus which includes a residue, usually A, corresponding to the 3' terminal U of the vRNA preceding the remainder of the virus-specific sequence (Krug, 1983; Figure 7). However, only newly synthesized mRNA can act as primer since the methyl groups at the 5' cap of viral mRNAs are labelled only if cells are incubated with ^{3}H-methyl methionine after, but not before, infection (Herz *et al.*, 1981) and addition of α-amanitin during infection inhibits mRNA synthesis (Mark *et al.*, 1979). Heterogeneous nuclear RNAs act as primers but it is not known whether nascent or mature forms are utilised (Krug, 1983). If nascent forms were utilised this would explain why only newly synthesized transcripts act as primers but this observation could equally be the result of rapid transport of suitable primers from the nucleus. The poly A tails of mRNAs synthesized *in vivo* are shorter and more homogeneous in length than those of transcripts produced *in vitro* (Plotch and Krug, 1977).

Transcription *in vivo* can be arbitrarily separated into two phases depending on the sensitivity to cycloheximide (CYH). In the presence of this drug transcription directed by the input virus takes place but vRNA synthesis and amplified transcription that constitute the secondary phase are prevented (Hay *et al.*, 1977; Barrett *et al.*, 1979). Secondary transcription is therefore dependent on protein synthesis. During primary transcription

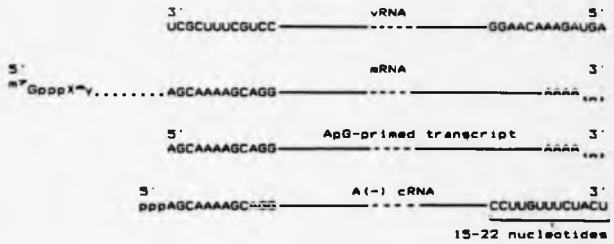


Figure 7: Diagram to show the relationship between influenza vRNA and transcripts.

Diagram to show the relationship of vRNA (- sense) and + sense transcripts. The sequences common to each of the genome segments are indicated. $AAAAA_n$ denotes poly A tail, and the 11-14 nucleotides donated by heterologous mRNAs to influenza mRNAs is indicated by a dotted line. The 5' terminal AG of the ApG-primed fragment is the incorporated ApG. Adapted from Lamb and Choppin (1983).

similar relative amounts of the different viral mRNAs are synthesized (Hay et al., 1977; Barrett et al., 1979) but during secondary transcription a second type of positive-sense cRNA is produced (Hay et al., 1977; Hay et al., 1982). These are full-length copies of the genome including the 17-22 nucleotides not copied into mRNA, which in turn act as template for the synthesis of progeny genomes (Figure 7). A primer is not required, nor are these transcripts polyadenylated and these antigenomes are therefore co-terminal with the virion RNA and the complement of the complete genetic information of the virus (Hay et al., 1977).

Anti-terminator activity is required to read through the poly-adenylation signal and this function is probably performed by NP. Depletion of free NP (NP not associated with nucleocapsids) from a cell lysate abolished synthesis of full-length transcripts (Beaton and Krug, 1986). Depletion of NS1 had no effect, but other viral proteins may be involved.

The viral proteins responsible for RNA replication have not been identified, but studies with temperature-sensitive mutants implicate a role for PB1 and PA (reviewed by Mahy, 1983). The polymerase proteins may be modified in some way to produce a replicative enzyme complex (Krug, 1983).

(c) Cellular site of transcription and replication

Influenza virus mRNA, template RNA and vRNA synthesis takes place in the nucleus (Krug, 1983; Kingsbury, 1985 for reviews; Enami et al., 1985; Shapiro et al., 1987). Pulse-labelled viral RNA

transcripts are found in association with a sub-nuclear structure, called the nuclear cage, with which newly synthesized cellular RNA transcripts are also associated (Jackson et al., 1982). Influenza viral mRNAs, in common with other mRNAs synthesized in the nucleus, are also methylated at internal A residues (Krug et al., 1976; Krug et al., 1980) probably as a result of nuclear enzymes. In addition, host enzymes are responsible for the splicing of transcripts from segments 7 and 8 (Lamb and Lai, 1982, 1984). After synthesis viral mRNAs are transported to the cytoplasm (Herz et al., 1981). Progeny genomes are transported to the cytoplasm after synthesis and encapsidation but template RNAs, also encapsidated, remain in the nucleus (Shapiro et al., 1987).

(d) Control of influenza virus gene expression

Immediately after infection all eight viral mRNAs are synthesized in similar amounts (Hay et al., 1977) and this is followed by the synthesis of approximately equimolar quantities of template RNAs (Shapiro et al., 1987). Subsequently two phases can be delineated during which the synthesis of the relative quantities of each RNA species and gene products are regulated (reviewed by Krug, 1983; Enami et al., 1985; Shapiro et al., 1987). The early phase is characterised by the predominant synthesis of NS and NP vRNAs and NS1 and NP mRNAs and proteins. By contrast synthesis of M1 mRNA and protein and to a lesser extent HA and NA are delayed. Gene expression during this phase is under transcriptional control because the level of protein synthesis is determined by the quantity of mRNA (Hay et al., 1977; Smith and Hay, 1982) which in turn is proportional to the amount of vRNA (Shapiro et al.,

1987). The duration of the early phase varies with cell type and the end is marked by maximal synthesis of mRNAs (Shapiro et al., 1987).

There is disagreement over the interpretation of events occurring during the second phase of gene expression (Shapiro et al., 1987). The evidence available suggests that vRNA synthesis and mRNA synthesis are uncoupled. Viral RNA synthesis continued at the maximal rate, but the rate of mRNA synthesis decreased from the maximal rate at 2.5 hours post-infection to 5% of the maximum rate at 4.5 hours post infection (Shapiro et al., 1987). However, the rate of synthesis of viral proteins was not reduced, implying that mRNA synthesized during the early phase is directing translation requiring some form of translational control. This is illustrated most dramatically by the example of M1 in BHK-21 cells where the rate of mRNA synthesis peaks at 2.5 hours post-infection but protein synthesis is maximal at 4.5 hours post-infection (Shapiro et al., 1987). The nature of the control operating during the second phase remains contentious, however. Other authors concluded that expression remained under transcriptional control in the later phase because the rate of synthesis of NS1 protein of FPV declined as the rate of synthesis of all viral mRNAs decreased (Hay et al., 1977; Inglis and Mahy, 1979). At the same time however, the rates of synthesis of the other viral proteins increased to, and were then maintained at, the maximal levels (Inglis and Mahy, 1979; Skehel, 1972) inconsistent with transcriptional control (Shapiro et al., 1987).

B. ASSEMBLY AND RELEASE

Since the synthesis of vRNA takes place in the nucleus and vRNA is never found except associated with NP in nucleocapsids, the first stage of assembly probably occurs in the nucleus (see Dubois-Dalcq et al., 1984 for review) but there is no direct evidence. A selective mechanism must exist for the transport of vRNA-containing nucleocapsids from the nucleus since the antigenomes remain in the nucleus (Shapiro et al., 1987). M and NP appear by immuno-electron microscopy to associate loosely together in the nucleus (Patterson, S., Gross, J. and Oxford, J.S. personal communication). M1, or another M gene product, may be responsible for transport of nucleocapsids since lesions in this gene result in accumulation of nucleocapsids in nuclei (Enami et al., 1985).

The envelope components assemble at the surface. HA and NA become inserted into the plasma membrane (Lenard and Compans, 1974) and are probably recognised by an M gene product at budding sites (Lohmeyer et al., 1979; Bukrinskaya et al., 1981; Maeno et al., 1981). This interaction may induce curvature in the membrane and thereby trigger budding (Dubois-Dalcq et al., 1984). HA is not an obligatory requirement for production of virions. A conditional-lethal (temperature-sensitive) mutant gave rise to non-infectious particles at the non-permissive temperature which lacked spikes and were of lower density. These particles contained less than one HA molecule per virion and the NA content was enriched 2-fold (Pattnaik et al., 1986). Virions are almost devoid of host proteins (Holland and Klein, 1970; Garten and Klenk, 1983) and therefore some mechanism must exist for their

exclusion from budding sites prior to release.

The mechanism responsible for packaging nucleocapsids into particles is not understood, but nucleocapsids have been seen to align under the membrane (Dubois-Dalcq et al., 1984). Other workers (Patterson, S., Gross, J. and Oxford, J.S., personal communication) could readily detect HA in the cell membrane by immuno-electron microscopy, but only rarely detected M and NP on the cytoplasmic side of the membrane during virus release. This may indicate that budding proceeds rapidly. Influenza virus buds predominantly from the apical surface of polarised cells to which the glycoproteins are selectively routed (Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan and Pendergast, 1980; Fuller et al., 1984).

NEUTRALISATION OF ANIMAL VIRUSES BY ANTIBODY

The immune processes involved in recovery and protection from influenza virus infection are complex and no attempt will be made to summarise them here (see Ennis, 1982; Mitchell et al., 1985; Stuart-Harris et al., 1985; Ada and Jones, 1986, for reviews). Neutralisation is the loss of infectivity as a result of combination with antibody. In primary infection it may play a role in limiting viraemia and contribute to recovery. Neutralising antibody plays a role in protection against reinfection and transfer of antibody can confer passive protection.

For many years the dogma was that neutralising antibody prevented attachment of viruses to cells. It is now realised that this is only one of a number of mechanisms and the study of these mechanisms also yields information about virus-cell interactions and virion structure.

Each of the components of the neutralisation reaction, namely antibody, virus and the host cell, plays a role in determining the outcome and these components are considered first.

9. ANTIBODY STRUCTURE AND PROPERTIES

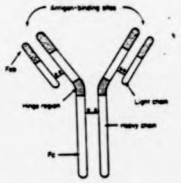
Antibodies or immunoglobulins (Ig) are glycoproteins produced by B-lymphocytes, now considered to be products of a supergene family that includes T-lymphocyte receptors (see Williams, 1984; Hood et al., 1985 for reviews).

There are five classes of Ig namely IgG, IgM, IgA, IgD, and IgE and the first three of these have been shown to cause

neutralisation. Figure 8 shows schematic diagrams of their structure and Table 1 summarises some of their properties. The basic Ig structure is exemplified by IgG (Figure 9) and consists of four polypeptide chains (two pairs) which form a Y-shaped structure comprised of two identical heavy (H) and two identical light (L) chains (see Porter, 1959; Nisonoff et al., 1975; Burton, 1985 for reviews). H and L chains are held together by strong non-covalent forces and usually at least one interchain disulphide bond, and similar interactions are responsible for the H-H chain association.

Each H and L chain consists of constant and variable regions. There are five classes of C_H which define the Ig class and each is functionally and antigenically distinct. The C_H regions that comprise the Fc region have important effector functions including complement fixation and placental passage. Some cells, e.g. phagocytes, bear receptors for the Fc region which can result in enhanced uptake of virus-antibody complexes. In mammals there are two types of light chain, called kappa and lambda, which can be linked to H chains of any class. The constant regions vary slightly within a class for example four sub-types of IgG in man have been defined.

The antigen-binding site or paratope is formed by the juxtaposition of two variable N-terminal regions, one each from heavy and light chains (V_H and V_L respectively). Consequently each four chain unit has two antigen binding sites. A huge diversity of specificities is generated from a relatively small number of genes by a process of recombination and mutation. In germ line cells the Ig genes are separated along the chromosome and include multiple copies of V_H and V_L sequences. During maturation of the B-lymphocyte the genes for a complete Ig



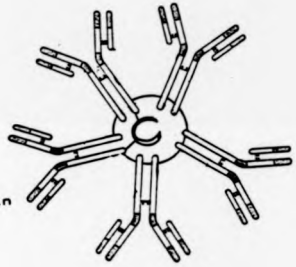
IgG

Heavy chain = γ

sIgA

Heavy chain = α

Structure includes secretory component and J chain.



IgM

Heavy chain = μ

= J chain

Figure 8: Schematic diagrams of the structure of Ig molecules. Information from Nisonoff et al. (1975).

Table 1: Summary of the properties of the immunoglobulins involved in anti-viral responses in man

Property	Immunoglobulin class			
	IgG	IgM	IgA (a)	sIgA
Approx. mol. wt.	150,000	900,000	160,000	385,000
Heavy chain				
Structure	4 ch. unit	(4 ch. unit)$_n$ + J ch.	4 ch. unit	(4 ch. unit)$_n$ +J ch. and Sc
Valency	2	10	2	4
% total Ig in serum	80	6	13	neg.
Subclasses	1,2,3,4	1,2	1,2	1,2
Complement fixation	+(b)	+	-	-

4 ch. unit = basic $(LH)_2$ molecule.

(a) Also present in very low concentrations in serum as a dimer polymerised by J chain only, total mol. wt. 335,000.

(b) The IgG4 sub-class of human IgG is not capable of activating complement.

neg = negligible; Sc = secretory component.

n.b. concentration of immunoglobulin (Ig) may vary in individuals.

Information from:

Nisonoff, A. (1984) "Introduction to Molecular Immunology"; Ed.II, Publ. Sinauer assoc. Sunderland, Mass.; Roitt, I.M. (1985) "Essential Immunology" Ed.V, Publ. Blackwell Sci. Publ., Oxford, London and Edinburgh and Taylor (1986).

consisting of sequences encoding constant, variable, joining and diversity regions are brought together by recombination and in the process somatic mutations in the N-terminal regions occur at a high rate (see Tonegawa et al., 1983 for review).

(a) IgG

IgG is the most abundant Ig in normal human serum and is the class of antibody predominantly stimulated by vaccination. Sub-classes of IgG exist, the number being dependent on species: four in mouse and man and two in rabbits. The relative abundance of the sub-classes in serum varies as do the biological activities of their Fc regions (Oi et al., 1984).

IgG can be cleaved proteolytically into fragments that maintain some of the functions of the intact molecule (Porter, 1959; Nisonoff et al., 1975; Putnam et al., 1962). Under reducing conditions papain cleaves on the N-terminal side of the disulphide bond that holds together the H chains yielding two monovalent Fab (antigen binding) fragments and the Fc (crystallisable) fragment. Fab fragments are not able to cross-link separate antigens and therefore cannot form aggregates. Pepsin acts on the C-terminal side of the H-H disulphide bridge producing a bivalent $F(ab')_2$ fragment and a partially degraded Fc region. Reduction of the disulphide bond linking the remainder of the heavy chains produces monovalent Fab' fragments (Figure 10).

(b) IgM and IgA

IgM and IgA are both found as polymers of the basic Ig unit. IgM is the first antibody class produced by mammals in response to

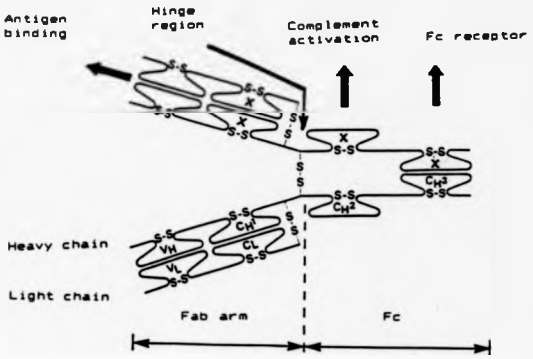


Figure 9: Diagrammatic structure of IgG

Showing the domains of the light and heavy chains and intra- and inter-chain disulphide bonds. The Fc region forms the stem of the molecule and the hinge region links this to the antigen binding Fab arms. There are two heavy and two light chains. The heavy chains have three constant domains and one variable domain. The light chains have one constant and one variable domain. Together the variable regions form the antigen binding sites (or complemetarity determining regions).

See text for references.

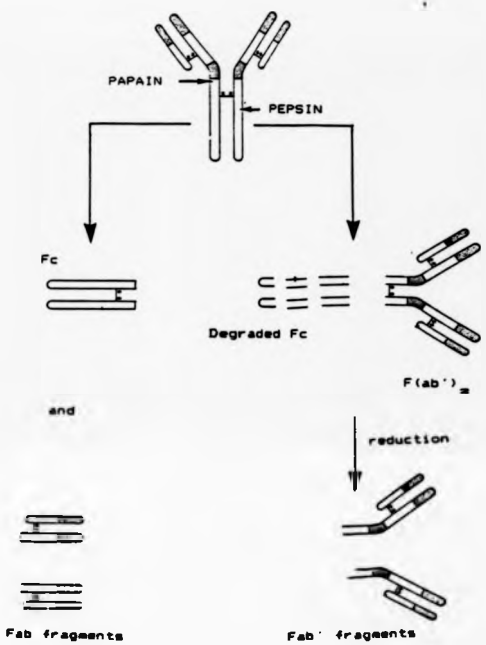


Figure 10: Fragmentation of IgG with Papain or pepsin

See text for references.

antigen. It consists of five, four chain Ig units and a J chain (15,000 mw) that initiates polymerisation. IgM has a valency of 10 (all paratopes within a pentamer are identical) and is a potent activator of the classical complement pathway. IgA exists in two forms. In human serum the predominant form of IgA is monomeric, whereas in mucosal secretions dimeric secretory IgA (sIgA) is more abundant, but there is species variation in the distribution of Ig classes in secretions (Tomasi, 1976). The presence of sIgA in secretions is due to local production and this dimeric form is composed of two four chain units, J chain and a secretory component.

10. THE IMPORTANCE OF ANTIBODY VALENCY TO NEUTRALISATION

The combination of virus (V) and antibody (Ab) occurs in two stages (Mandel, 1979; 1985 for reviews) each distinguishable by the ease with which the virus-antibody complex dissociates:

$$V + Ab = (VAb)_r \qquad (1)$$

$$(VAb)_r = (VAb) \qquad (2)$$

Where: $(Vab)_r$ = freely dissociable virus-antibody complexes

(Vab) = more stable virus-antibody complex

The ease of dissociation decreases with time, but is influenced by temperature. Stage 1 occurs rapidly, irrespective of temperature and stage 2 is slow at 0°C but at 37°C stable virion-antibody complexes form in minutes. The rate of the reaction is favoured by higher temperature which increases the number of collisions and facilitates shifting of complementary regions to the most stable position, but a decrease in temperature favours complex formation because the reaction is

exothermic. The relative contributions of entropy and enthalpy may be temperature dependent with the forward reaction being driven at low temperature by the enthalpy change and at high temperature by the change in entropy (Szeczuk and Mukkur, 1977).

Antibody affinity is the strength of interaction with a single antigenic determinant and reflects the stereocomplementarity of epitope and paratope. The complex is held together by non-covalent, short-range electrostatic and van der Waals forces and may therefore dissociate. For example binding of Fab fragments to antigen is dependent on affinity and stable complexes form if the affinity is sufficiently high. Antigen-antibody complexes are however orders of magnitude more stable. If bivalent anti-HA IgG binds to two epitopes on the same HA spike or on adjacent spikes of the same virion, dissociation of the complex can only occur in the unlikely event of both Fab arms dissociating simultaneously. Therefore, the higher the valency, the more stable the complex formed by antibody of a given affinity, this is called avidity. The valency and or size of dimeric sIgA and pentameric IgM may contribute to neutralisation, as demonstrated for influenza virus (Taylor and Dimmock, 1985a,b). Thomas et al. (1986) showed that when poliovirus is neutralised by a monoclonal IgM very few small aggregates are formed compared with virus reacted with IgG, suggesting that the valency of IgM favours formation of large aggregates.

11. THE VIRAL COMPONENT

Antibody causes neutralisation by binding to a site on virus which inhibits a process required for infectivity. Daniels (1975) termed such a site a critical site and Mandel (1979) defined loss

of infectivity in this way as primary, intrinsic neutralisation. In this thesis the mechanism of neutralisation of influenza virus by antibody alone is considered. The neutralising antigen, to which neutralising antibody binds, may consist of a number of neutralisation sites and the mechanism of neutralisation may depend on the epitope. The neutralisation antigen of influenza virus is the HA trimer and the neutralisation sites are localised on the globular head. Antibodies to other sites on the HA (Breschkin et al., 1981) and to the other surface glycoprotein, the NA, can reduce infectivity only by aggregation at suitable concentrations (Seto and Rott, 1966; Webster and Laver, 1967; Kilbourne et al., 1968; Compans et al., 1969). Neutralisation may require a third factor, for example complement, and is then considered to be the result of mediated intrinsic neutralisation (Mandel, 1979).

Under certain circumstances, loss of infectivity can result from antibody binding to non-neutralising sites and Mandel (1979) termed this extrinsic neutralisation. This non-neutralising antibody can mediate virolysis in the presence of complement. Pseudoneutralisation of infectivity by aggregation of a number of virions into one infectious unit is an extrinsic neutralisation mechanism.

In practice these theoretical distinctions may not be realistic, for example the infectivity of virions in aggregates may also be inactivated. Antibody-mediated neutralisation results in abrogation of virus-cell interactions either by steric interference or inducing a change that renders the virus incapable of initiating infection.

12. KINETICS AND STOICHIOMETRY OF NEUTRALISATION

The weight of evidence from studies of the kinetics of neutralisation suggests that the reaction is first-order, implying that infectivity is destroyed by a single antibody-virus interaction (single-hit). This has to be reconciled with the presence of many VAPs on virions and measurements of the number of antibody molecules required to neutralise infectivity.

Kinetic experiments are performed by mixing virus and antibody and assaying samples for infectivity as incubation proceeds. A first-order reaction is characterised by an immediate, exponential decrease in infectivity. In time the rate decreases leaving an non-neutralised or persistent fraction (see Part 14, below). The curve produced by higher-order reactions includes a delay or lag period in proportion to the order of the reaction.

For a variety of animal viruses kinetic studies show no lag period and infectivity is lost at a constant rate for a set period of time, characteristic of a first-order reaction (for review and early references see Mandel, 1979; Icenogle *et al.*, 1983a; Wetz *et al.*, 1986; Taylor *et al.*, 1987). However, higher-order kinetics were seen under critical conditions. Incubation of influenza virus at low temperature (0°C) with low concentrations of polyclonal antibody produced a lag period (Lafferty, 1963a). Studies using polyclonal antibodies are difficult to interpret because of the mixture of paratopes present. However, similar results using low concentrations of monoclonal anti-HA IgG at 4°C were also obtained by Taylor *et al.* (1987), but the reaction was at most second-order (two-hit). A lag was also seen when poliovirus was neutralised by IgM (Phillipson, 1966).

Combination of antibody with haptens occurs in less than 3 seconds (Pecht et al., 1972) and therefore kinetic studies of neutralisation may appear first-order because of inability to sample the reaction sufficiently quickly. Even so, neutralisation appears from kinetic evidence to result from the binding of one (or at most a few) antibody molecule(s) making the theory that neutralising antibody prevents attachment to host cells untenable unless there are only a few attachment sites. The envelopes of animal viruses are composed of a number of identical viral attachment proteins (VAP) carrying viral attachment sites (VAS) each of which can interact with a cell receptor unit (CRU). Influenza virions have 700-1,000 HA trimers and each monomer carries a VAS. With 2,100-3,000 VAS per particle it would appear impossible for one or a few antibody molecules to prevent attachment. The poliovirus virion is composed of 60 protomers (Rueckert, 1976) and each picornavirus protomer is thought to carry a VAS (Rossman et al., 1985; Hogle et al., 1985). Since neutralisation by 67% is achieved by 4 antibody molecules per particle it is the binding of antibody to a few critical sites on the virion which leads to neutralisation.

Studies of the number of antibodies bound to neutralised virions show that more than one or two molecules of antibody are required. Sub-neutralising quantities of some monoclonal antibodies to Sindbis virus and West Nile virus (WNV) cause enhancement (Chanas et al., 1982; Peiris et al., 1982). Antibody binds to HSV (Ashe and Watkins, 1967) and Venezuelan equine encephalitis virus (VEEV) (Mahon, 1970) before neutralisation occurs, allowing mediated neutralisation by anti-Ig, but this cannot be physiologically important.

Direct quantitation of the number of antibodies bound has been carried out for influenza virus and poliovirus. Influenza virus binds a maximum of one anti-HA IgG per spike whether monoclonal (3 epitopes to which antibody could bind per trimeric spike) or polyclonal (12 epitopes per spike) is used (Taylor et al., 1987). However, neutralisation by 67% (1/e) is brought about by 70 molecules of either of two monoclonal antibodies that bind to different epitopes (Taylor et al., 1987). To reconcile this finding with the one- or two-hit kinetics, these authors suggest that the antigenically identical HA spikes can be divided functionally into neutralisation relevant and neutralisation irrelevant spikes. Only antibody binding to the neutralisation relevant spikes, which are in the minority, reduces infectivity. In support of this, infectious virus was found in association with neutralising monoclonal antibody.

For poliovirus the number of molecules of monoclonal (Icenogle et al., 1983) or polyclonal (Wetz et al., 1986) antibodies required to effect neutralisation was found to be different. Icenogle et al. (1983) determined that a maximum of 30 monoclonal IgG molecules could attach to each virion, but only 4 molecules per virion were required to reduce infectivity by 67%. These authors suggested two explanations for their results. The "some sites critical" model is that favoured by Taylor et al. (1987) for influenza virus and proposes that only one in four of the antibodies reduced infectivity i.e. that only one in four sites to which this antibody binds mediate neutralisation. Thus, an average of one molecule of antibody per virion will inactivate 25% of the virions in a population. The other theory with which their results are compatible is a step-wise model where all the epitopes are critical, but binding of each antibody molecule reduces the infectivity by a factor of 3/4. The former

explanation appears more likely as it is hard to envisage a fraction of an infectious unit. With a polyclonal antibody, Wetz et al. (1986) found that infectivity was reduced by 20% when a single antibody molecule was bivalently attached to one in five virions. These results could be explained in terms of either of the Icenogle et al. (1983) models. The polyclonal antibody of Wetz et al. (1986) and the monoclonal antibody may bind to different epitopes and the epitopes to which the polyclonal antibody binds may all mediate neutralisation. Wetz et al. (1986) suggest that avidity may be important and by the step-wise model, the (hypothetically) greater avidity of the polyclonal IgG may result in reduction of infectivity by 100% rather that 25%. The issue remains to be resolved.

13. THE INFLUENCE OF THE HOST CELL ON NEUTRALISATION

The third variable in the neutralisation reaction is the host cell used to assay residual infectivity. Kjellen and Schlesinger (1959) neutralised VSV with a polyclonal antibody and found that the neutralisation titre was up to 10^{2}-times higher on CEF cells compared to that obtained on cells derived from leukaemic mouse bone-marrow. Differences in the extent of neutralisation of echovirus type 4 by polyclonal antibody have also been reported when assayed on green monkey kidney cells or rhabdomyosarcoma (RD) cells (Kjellen and von Zeipel, 1984).

Grady and Kinch (1985) investigated the ability of a panel of monoclonal IgGs to neutralise La Crosse virus (LAC). One IgG neutralised infectivity in BHK-21 cells but not Aedes albopictus (mosquito) cells and a different IgG was opposite in effect. Each of the IgGs recognised epitopes in distinct regions of the G1 glycoprotein.

Steric considerations may explain these observations. Influenza virus neutralised by IgG fails to bind to red blood cells (RBCs) but attaches normally to a number of types of cultured cells (Dimmock et al., 1984). Figure 11 shows the relative sizes of the HA spike (VAP), IgG and glycophorin, the putative CRU for influenza virus on RBCs. If the CRU of tissue culture cells is sufficiently long then it will be able to reach the VAS which is distinct from the antigenic sites to which the antibody is bound.

For enterovirus 71 a more complicated example of host-dependent neutralisation has been described (Kjellen, 1985). By adsorption with protein A it was found that polyclonal antiserum was a mixture of populations. One population reduces infectivity when virus is treated with antibody and the mixture inoculated onto cells. The other neutralises virus only after adsorption to cells and was only effective on one of two cell types tested. This suggests that new antigenic sites are revealed when the VAP binds to the CRU (Figure 12).

These studies suggest that it is important to consider the interaction of neutralised virus with differentiated cells to understand the mechanism of protection in the host animal and may explain the results of Buchmeier et al. (1984). These workers found that a monoclonal antibody that neutralised infectivity of murine hepatitis virus type 4 in cultured L-24 cells failed to passively protect mice against challenge, whereas another antibody of the same sub-class was both neutralising and protective.

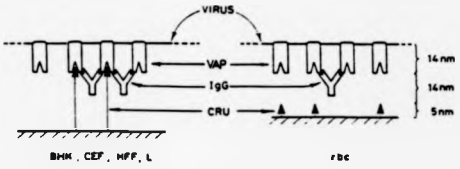


Figure 11: Hypothetical scheme to explain how IgG blocks attachment of influenza virus to erythrocytes but not other cells.

From Dimmock, 1987.

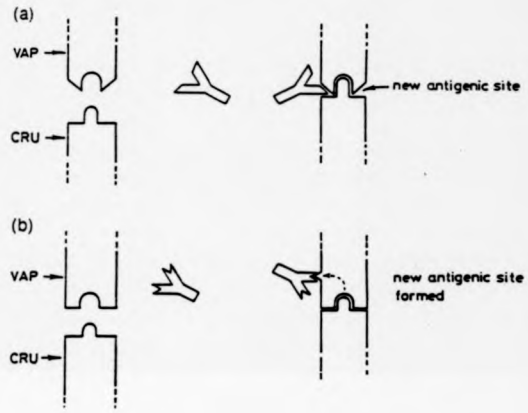


Figure 12: Possible explanations of the cell-dependent neutralisation of enterovirus 71 through the creation of new antigenic sites either (a) directly or (b) indirectly.

From Dimmock, 1987.

14. THE NON-NEUTRALISED (PERSISTENT) FRACTION

The residual infectivity after incubation of antibody and virus is called the non-neutralised or persistent fraction (Dulbecco et al., 1956). Like neutralisation, the magnitude of the persistent fraction is determined by properties of antibody, virus and host cell. Neutralisation induced by low-avidity antibody is dependent on the dilution of antibody-virus complexes (Lafferty 1963a,b). When a virus is treated with polyclonal antibody, non-neutralising and neutralising antibodies may compete for binding if the epitopes are sufficiently close (Dulbecco et al., 1956). Direct evidence for this type of effect was provided by Massey and Schochetman (1981) using monoclonal antibodies. Non-neutralising antibody bound to Kirsten sarcoma virus prevented subsequent attachment of neutralising antibody and protected the virus from neutralisation. The persistent fraction of herpes simplex virus was further neutralised by Fab when intact IgG could cause no increase in neutralisation, presumably because of the smaller size of the fragments (Ashe and Notkins, 1967).

The ability to select escape mutants from virus populations in the face of neutralising antibody attests to the presence of antigenic variants resistant to neutralisation. For echovirus type 4 it has been suggested that the presence of virus aggregates protects a section of the population from neutralisation. Aggregates were removed by filtration and the persistent fraction disappeared (Wallis and Melnick, 1967). Later this was disputed by Kjellen and von Zeipel (1984) who demonstrated that incubation of virus with disrupted filters was sufficient to reduce the persistent fraction. Filtration reduces the size of, but does not eliminate, the persistent fraction of

VEEV (Hahon, 1970) and poliovirus (Lewenton-Kriss and Mandel, 1972).

The influence of the host cell on the neutralisable fraction (and therefore conversely the non-neutralisable fraction) has been described (Part 12, above).

15. MECHANISMS OF NEUTRALISATION OF VIRUS BY ANTIBODY

Antibody-mediated neutralisation mechanisms have been comprehensively reviewed by Dimmock (1984; 1987) and Mandel (1979; 1984; 1985). The information available demonstrates that neutralising antibody can cause the interruption of any of the events required for initiation of infection and, for a given combination of antibody, virus and cell, more than one mechanism may contribute to the loss of infectivity.

(a) Aggregation

Polymerisation of virions into aggregates reduces the number of infectious units and was defined by Mandel (1979) as pseudoneutralisation, an extrinsic mechanism. Aggregation is included here because some evidence suggests that virus in aggregates is inactivated and it is important in the neutralisation of poliovirus type I and adenovirus.

Neutralisation in proportion to aggregation occurs when adenovirus is neutralised by two polyclonal antisera containing anti-fibre specificities, but 3 to 5-times more antibody-treated virus binds to cells than untreated virus (Wohlfart *et al.*, 1985). During a normal infection adenovirus is destabilised after

interaction with the cell surface such that the viral DNA becomes sensitive to deoxyribonuclease (DNase) digestion. Adenovirus treated with anti-fibre antibody did not become susceptible to DNase, 85% of the virions remained attached to the surface of HeLa cells and the 15% that penetrated into cells remained as aggregates in vesicles (Wohlfart et al., 1985). Neutralisation of adenovirus by antibodies to the fibre therefore appears to be the result of formation of aggregates which in turn are not able to penetrate into cells. This is not the only mechanism by which infectivity of adenovirus is reduced because, as described below, antibodies to the hexon protein do not aggregate adenovirus or prevent penetration.

Thomas et al. (1986) have concluded that aggregation alone accounts for neutralisation of poliovirus type I. These workers showed that six monoclonal IgGs, one monoclonal IgM and two polyclonal antibodies neutralised virus and caused aggregation. The relative quantities of antibody and virus were shown to be important: aggregation occurred when antibody was present in moderate excess. Virus was neutralised by 90-99% and the residual infectivity was correlated with the quantity of free virus remaining after antibody treatment (Thomas et al., 1985; 1986). Antibody was not associated with the free virus, but for aggregation alone to be responsible for the loss of infectivity predicts that single virions that have bound antibody, and the virions in aggregates, will be infectious. Supportive evidence comes from Icenogle et al. (1983) who show that 4 antibody molecules are required to neutralise infectivity (that is, neutralising antibody can bind to poliovirus without significantly reducing infectivity) and Brioen et al. (1983) who measured the infectivity of dimers, trimers and higher polymers. Conversely, Wetz et al. (1986) report that bivalent binding of

one molecule of polyclonal IgG per virion neutralised poliovirus and Thomas et al. (1986) reported that oligomers and aggregates formed with the same antibody as used by Brioen et al. (1985) do not contribute to the residual infectivity, implying that virions are both aggregated and inactivated. Another monoclonal antibody (1c) used in excess of the concentration required to cause aggregation and 99% neutralisation yielded non-infectious, mono-dispersed virion-antibody complexes (Thomas et al., 1986). Additionally, data of Mandel (1967a,b) and Emini et al. (1983a) demonstrate that poliovirus type I neutralised by more than 99% attaches to and penetrates cells (see below).

(b) Inhibition of attachment

Attachment of virus to cells is inhibited if antibody reduces the efficiency of binding of the VAP and CRU (Figure 13). Inhibition of attachment has been implicated as contributing to the neutralisation of a number of viruses but often accounts for only part of the loss of infectivity. For example, reduction of the infectivity of influenza virus by 98.5% with IgM inhibits attachment by only 50% (Taylor and Dimmock, 1985a).

The only example of inhibition of attachment of virus where the concentration of antibody used is known to be less than that required for saturation is poliovirus type I where four monoclonal antibodies to the same site prevent attachment (Emini et al., 1983a). Results for only one of these (I CJ27) are shown and under non-saturating conditions attachment was inhibited by 80%, but the neutralisation titre was not given. In a similar way, only some neutralising monoclonal IgGs to Venezuelan equine encephalitis virus (VEEV) neutralise by inhibiting attachment (Roehrig and Mathews, 1986). Attachment was inhibited by 90% but

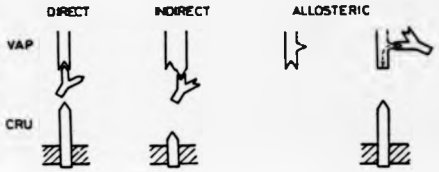


Figure 13: Possible mechanisms by which attachment of virus to the CRU is inhibited by antibody.

From Dimmock, 1987.

it is likely that excess antibody was used (10 µg IgG/ 5 µg virus). The extent of neutralisation and the effect of smaller amounts of antibody were not reported.

In other studies inhibition of attachment is, or appears to be, dependent on the use of excess antibody. Attachment of Newcastle disease virus (NDV) to CEF cells was prevented by quantities of polyclonal antiserum but only in excess of those required to neutralise infectivity (Rubin and Franklin, 1957). Monoclonal antibodies to herpes simplex virus type 1 (HSV-1) that are effective in preventing attachment have only weak neutralising activity. Additionally, potent neutralising IgGs had little effect on attachment at concentrations significantly higher than required for neutralisation (Fuller and Spear, 1985). Lee et al. (1981) demonstrated that the VAP for reovirus is the σ1 protein and monoclonal IgGs to the σ1 protein and λ2 protein prevent attachment to mouse L-fibroblasts. It is probable that virus was reacted with an excess of antibody (40 µg of monoclonal IgG, not all of which may be virus-specific, per 10 µg of virus) but the quantitative relationship between prevention of attachment and neutralisation was not considered. Consequently the data are difficult to assess.

Prevention of attachment contributes to neutralisation of visna virus and influenza virus. Attachment of visna virus to sheep choroid plexus fibroblast cells after neutralisation with immune serum raised in a sheep, was inhibited by 80%. (Kennedy-Stoskopf and Narayan, 1986) Attachment of influenza virus is inhibited only by polymeric antibody. Secretory IgA (sIgA) prevents attachment of influenza virus to BHK-21 cells at 4°C, and at 25° and 37°C 50% of virus neutralised with sIgA or IgM failed to attach (Taylor and Dimmock, 1985a,b).

Similarly, 19S antibody (IgM) reduced attachment of

poliovirus type I to HeLa cells by between 25-75% of the control (Mandel, 1967a). The majority of neutralising IgGs to poliovirus type I allow attachment and penetration, but monoclonal antibodies to one epitope completely prevent attachment at non-saturating but neutralising concentrations (Emini et al., 1983a). In some cases neutralising antibody increases attachment of virus to cells for example poliovirus type I (Mandel, 1967a; Emini et al., 1983a), NDV (Silverstein and Marcus, 1964), rabbitpox virus (Joklik, 1964) and VEEV (Roehrig and Mathews, 1986). For poliovirus this effect was non-specific as attachment to cells lacking receptors for the virus was also increased (Emini et al., 1983a).

(c) Inhibition of events after attachment

Many studies demonstrate the attachment of neutralised virus to cells without identifying the subsequent stage at which infection is interrupted. Some examples have already been given. Potent neutralising antibodies to glycoprotein D of HSV-1 allow attachment to Hep2 and Vero cells (Fuller and Spear, 1985) and some monoclonal IgGs to VEEV fail to prevent attachment of 40-50% of neutralised virus (Roehrig and Mathews, 1986). NDV neutralised with polyclonal antibody attached to CEF cells (Rubin and Franklin, 1957) and monoclonal antibodies to the haemagglutinin-neuraminidase (HN) and fusion (F) protein can neutralise infectivity after adsorption of NDV to MDBK cells (Russell, 1986). Neutralised virus may attach to cells by abnormal mechanisms for example neutralised VSV attaches to a receptor not normally used by the virus (Schlegel and Wade, 1983) and IgG may act as a VAP when neutralised virus is inoculated onto cells with Fc receptors.

(d) Inhibition of penetration

Penetration of virus into cells is thought to involve formation of a cell receptor site (CRS) by nucleation of a number of CRUs (Figure 14) as occurs in receptor-mediated endocytosis. Thus, antibody may prevent penetration by interfering with nucleation (Dimmock, 1987).

Penetration can be assayed by incubating virus and cells and attempting to remove virus-antibody complexes with a suitable agent, for example, proteases (Helenius et al., 1980), urea (Eminí et al., 1983a) or receptor destroying enzyme (RDE) (Rubin and Franklin, 1957). Using proteinase K, Taylor and Dimmock (1985a,b) were able to remove the 50% of influenza virus that attached to BHK-21 cells at 25° and 37°C after neutralisation with sIgA or IgM, demonstrating that these antibodies prevent penetration. Nguyen et al. (1986) showed that transmissible gastroenteritis virus (TGEV) attaches to, but does not penetrate, swine testis or pig kidney cells when neutralised by polyclonal sIgA or IgG. As described above, 85% of adenovirus aggregated by anti-fibre antibody failed to penetrate into HeLa cells (Wohlfart et al., 1985). A hyperimmune serum and some monoclonal IgGs prevent penetration of poliovirus type I into HeLa cells (Emini et al., 1983a).

(e) Inhibition of uncoating

Uncoating is a general term for the ill-defined events between penetration and the first virus-specific synthetic event, transcription or translation of the input genome. West Nile virus (WNV) enters cells by receptor-mediated endocytosis involving a low pH fusion reaction in endosomes of P338D1 cells (Gollins and

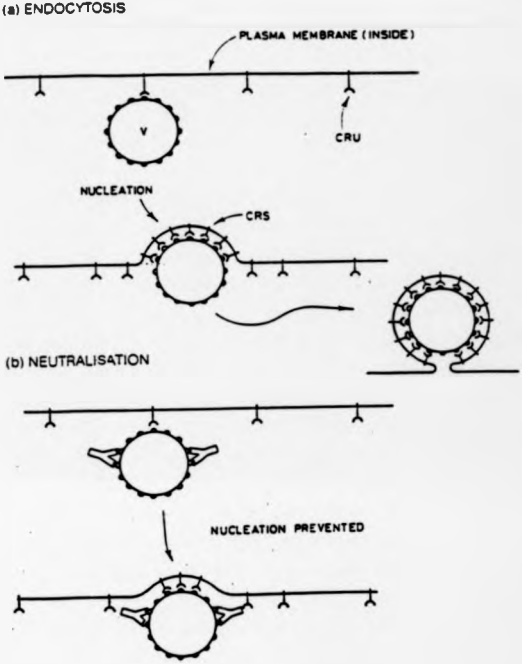


Figure 14: Scheme suggesting how antibody could neutralise virus (b) by preventing the normal nucleation of cell receptor units (CRUs) to form a functional cell receptor site (CRS)(a).

From Diamock, 1987.

Porterfield, 1984; 1985; 1986a). Uncoating is thought to be a result of fusion with endosomes and is accompanied by an increase in the susceptibility of the input genome to digestion by RNase. Gollins and Porterfield (1986b) showed that treatment with increasing amounts of neutralising polyclonal antibody progressively reduced the RNase sensitivity of [^{3}H]-uridine-labelled WNV in parallel with the loss of infectivity. The same antibody also prevented WNV-liposome fusion suggesting that WNV is neutralised by inhibition of the intra-endosomal fusion event. Studies of the HA of influenza virus have demonstrated that the conformational change triggered by mildly acidic pH that causes membrane fusion (General Introduction, Part 4) is prevented *in vitro* by neutralising antibody (Kida *et al.*, 1985; Wharton *et al.*, 1986). Interference with this conformational change *in vivo* could prevent liberation of influenza virus nucleocapsids from endosomes and has been proposed as a possible neutralisation mechanism, but without supportive evidence (Kida *et al.*, 1985). Failure to escape from intracellular vesicles of HeLa cells has also been implicated as contributing to the neutralisation of adenovirus (Wohlfart *et al.*, 1985). Penetration of adenovirus reacted with antibody to the hexon or penton base was as efficient as virus treated with a pre-immune serum, but approximately 69% and 47% respectively remained in vesicles as judged from electron micrographs. Anti-penton base antibody neutralised infectivity by no more than 50% and this correlated with the number of virus particles near to the nucleus (25% compared to 36 and 54% for controls) suggesting that the results obtained by this method were proportional to infectivity.

Rabbitpox virus neutralised with polyclonal antiserum fails to uncoat completely in HeLa cells (Joklik, 1964). Two-thirds of the

virus-antibody complexes that attach to cells elute, but the remainder penetrate into the cytoplasm. Infectious virions are converted to cores by removal of the envelope phospholipid (90% is degraded) and protein components, the latter probably by a virus-induced uncoating protein (Pedley and Cooper, 1987), rendering the viral DNA sensitive to DNase. The phospholipid component of neutralised rabbit pox virus is degraded but further uncoating, measured as sensitivity to DNase, does not occur (Joklik, 1964).

Mandel (1967b) proposed that neutralisation of poliovirus type I is the result of a defect in uncoating because virus neutralised by polyclonal IgG attached and penetrated into HeLa cells. Emini et al. (1983a) obtained similar results using monoclonal and monospecific antibodies, but showed that antibodies of one specificity prevented attachment (see above) and some antibodies inhibited penetration by up to 50%. However, the neutralised virus that entered cells did not direct transcription. Mandel (1967b) found uncoated, intact vRNA, thought to be a prerequisite for replication, in cells inoculated with infectious virus, but was unable to detect intact vRNA in cells inoculated with neutralised virus. This suggests that the vRNA of neutralised virus is degraded intracellularly.

(f) Inhibition of events after uncoating

There are reports of two viruses which uncoat normally by the measured criteria, but fail to replicate. When FPV/R is neutralised by 99.99% or more with polyclonal or monoclonal IgG, virus-antibody complexes attach to CEF cells, penetrate and the viral RNA becomes localised in the nucleus (Possee and Dimmock, 1981). Similar results were later obtained using BHK-21 cells and

virus neutralised with IgG or monomeric IgA (Taylor and Dimmock, 1985a). Influenza virus-IgG complexes also attach to human foreskin fibroblasts and L-cells with unaltered kinetics (Dimmock et al., 1984; Taylor, 1986). Additionally, Possee et al. (1982) demonstrated that the lipid envelope of neutralised virus remained in the cytoplasmic fraction of CEF cells. The kinetics of accumulation of virion RNA in the nuclei of CEF and BHK-21 cells were indistinguishable from those of infectious virus (Possee and Dimmock, 1981; Possee et al., 1982; Dimmock et al., 1984; Taylor, 1986) suggesting that neutralised virus enters cells by the same route as infectious virus. Viral RNA was apparently undegraded as judged by polyacrylamide gel electrophoresis (Possee, 1981). However, no viral transcription was detectable in cells inoculated with neutralised virus. It was also found that transcriptase activity in vitro of FPV/R and X-49 were reduced by 3 to 9-fold after treatment with neutralising antibody. Based on these findings it was proposed that neutralising antibody bound to the HA, and induced a conformational change that was transmitted across the viral membrane causing inhibition of the virion transcriptase enzyme (Possee, 1981; Possee et al., 1982).

Visna virus uncoating in transformed macrophages (TM) has been assayed by following the appearance of acid-soluble radioactivity in the tissue culture medium of cells inoculated with radiolabelled virus (Kennedy-Stoskopf and Narayan, 1986). In this cell line, uncoating of visna virus occurred after neutralisation by polyclonal antiserum, but replication did not occur, in contrast to SCPF cells where attachment is blocked. Attachment, penetration and uncoating of neutralised visna virus were all enhanced in TM compared with control virus, but virus RNA was not transcribed. Since macrophages possess receptors for the Fc

region of IgG and process antigen, antibody-treated visna virus may have been taken up by a non-infectious pathway and degraded.

(g) Co-operative neutralisation

The neutralising efficiency of some monoclonal antibodies is increased by the presence of other antibodies. Peiris et al. (1982) found that two monoclonal antibodies neutralised WNV poorly by themselves, but when pooled efficient neutralisation occurred. Effective neutralisation of La Crosse virus requires binding of monoclonal antibodies to at least two different but specific antigenic sites on the G1 glycoprotein (Kingsford and Ishizawa, 1984). Synergistic neutralisation of NDV has also been reported. Iorio and Bratt (1984) reacted virus with monoclonal antibodies to four different sites. Each antibody on its own failed to neutralise between 1-17% of the infectivity depending on the site recognised, but when all four were used together, neutralisation was equivalent to that obtained with a mouse polyclonal antibody. Enhanced neutralisation of NDV was obtained by Russell (1986) using monoclonal antibodies to different epitopes of the haemagglutinin-neuraminidase protein.

(h) Concluding remarks

For a number of viruses, including adenovirus, poliovirus and influenza virus, the same antibody can reduce infectivity by exerting an inhibitory effect at more than one stage in infection. For influenza virus the quantitative contribution of the different mechanisms has been assessed (Table 2). In this thesis the mechanism of neutralisation by IgG is addressed and Figure 15 shows schematically the stages at which different classes of antibody exert their effect.

Table 2: Summary of neutralisation of influenza virus by different classes of antibody

Ab	Stage of infection inhibited	Extent of inhibition (%)	Neutralisation titre (%)
Po* or Mo IgG	Transcription	100 (undetectable)	>99.9
Po IgA	Transcription ?	ND	98.5
Po sIgA	Attachment	50	>97
	Penetration	50	
PoIgM	Attachment	50	>99
	Penetration	50	

* Po = polyclonal, Mo = monoclonal.

ND = not determined

For refs. see text.

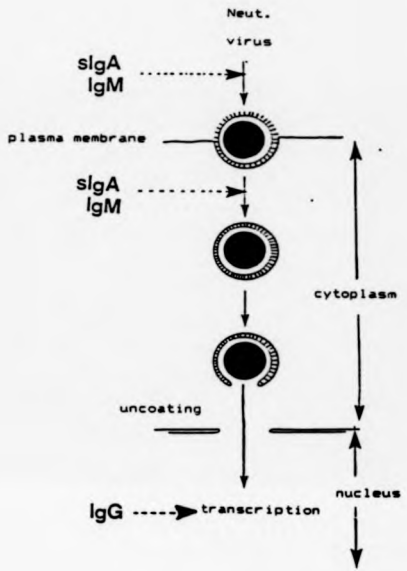


Figure 15: Diagram to show the stages at which different classes of antibody inhibit influenza virus infection.

This figure summarises the understanding of the mechanism of neutralisation of influenza virus before the present study.

16. CHANGES IN ANTIGENS AND VIRIONS INDUCED BY ANTIBODY

(a) Nature of antigenic determinants

Two types of epitope can be distinguished (van Regenmortel, 1986). Continuous epitopes are regions on the surface of proteins with distinctive conformational features made up of amino acid residues that are contiguous in primary sequence. Discontinuous epitopes are formed by the juxtaposition in space of residues that are not contiguous in the primary sequence. Consideration of continuous epitopes led to the suggestion that an epitope consisted of 5-8 amino acids (Atassi, 1975) but this represents an oversimplified view (van Regenmortel and Neurath, 1985). Two crystal structures have been solved for antigen-Fab complexes and in these 16 residues of the antigen are in close contact with a similar number of paratope residues. The estimated area of contact (the antibody footprint) is 20 x 30 angstroms (Amit et al., 1986; Colman et al., 1987). The antigenicity of part of a protein depends on a combination of accessibility, hydrophilicity and mobility, of which high mobility is the most reliable indicator of the antigenicity of a site (see Benjamin et al, 1984; Tainer et al., 1985; van Regenmortel, 1986, 1987; Williams and Moore, 1985 for reviews).

(b) Ig flexibility and function

Ig molecules are flexible and this property is thought to be important for antibody function (Figure 16). Flexibility about the hinge, formed of a sequence of 18 amino acids linking the C_H1 and C_H2 domains of IgG, allows movement of the Fab arms relative to each other (see Hansen et al., 1981; Davies and Metzger, 1983;

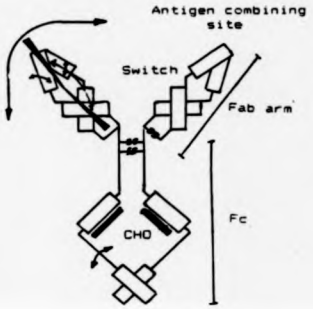


Figure 16: Diagram to show the extent of potential motion of different parts of the IgG molecule.

The extent of potential movement is indicated by double-headed arrows. Taken from Huber and Bennett (1987).

Huber and Bennett, 1987 for reviews).

The relative flexibility of Ig is related to the ability to fix complement (for review see Feinstein et al., 1986). Oi et al. (1984) produced a series of antibodies with different C_H classes and sub-classes by isolating mutant hybridoma cells. By a variety of means it was shown that higher flexibility at the hinge was reflected by increasing ability to fix complement and this was related directly to the ability to bind C1q, the part of the C1 complex that binds to antibody in the first event of the classical complement activation pathway.

IgG bound to antigen is found in a variety of shapes. In solution bound to monovalent hapten, the antigen binding sites of a hybrid rabbit antibody are at least 9 nm apart, the angle between Fab arms being 80-95° (Y-shape), assuming the Fab arms are 7 nm long (Werner et al., 1972). The shape can vary considerably, however. Valentine and Green (1967) showed by electron microscopy that when antibody binds hapten the angle between the Fab arms can vary between 10 and 180° (T-shaped). Wrigley et al., (1977; 1983a) present electron micrographs of polyclonal and monoclonal IgG bound to isolated glycoproteins of influenza virus and the antibody is seen in a variety of forms from Y-shape to T-shape with Fab arms at 180°. Thus, it is predicted that flexibility about the hinge allows binding of IgG to multivalent ligands, but the maximum distance that it is possible for an IgG to span is 14 nm. Lafferty and Oertilis (1963) showed IgG bound bivalently to influenza virions and, at higher concentrations of antibody, cross-linking particles. It is possible, though there is no supportive evidence, that flexibility could influence the ability to form aggregates. In addition to flexibility about the hinge,

Wrigley et al. (1983a) demonstrated that Fab arms can rotate about their long axis relative to the Fc region and that the arms can bend at the elbow or switch region between the variable and constant domains (Figure 16).

The three-dimensional structure of Fab NC41 and influenza virus NA suggests that a change is induced in the relative positions of V_H and V_L altering the arrangement of the segments involved in binding antigen (Colman et al., 1987; Huber and Bennett, 1987). The evidence is indirect as the structure of the uncomplexed Fab has not been solved. The complex of lysozyme with D3.1 Fab shows no similar conformational alterations, possibly because none are required (Amit et al., 1986). The site recognised by this Fab is not one of the antigenic sites proposed from studies using polyclonal antibodies or predicted from theoretical considerations (Tainer et al., 1984) and there is strong complementarity between paratope and epitope which seem to interact in a way similar to a lock and key.

(c) Conformational changes induced in antigen by antibody

The structure of the NA-NC41 Fab complex provides evidence for perturbations having occurred in the epitope (Colman et al., 1987). The epitope is composed of residues from four loops with above average mobility and the authors suggest that the paratope-epitope interaction is more like a handshake with paratope and epitope residues shifting as they come together rather than remaining rigid in a lock and key-type interaction.

Protein molecules are inherently flexible and exist in a state of perpetual motion about a mean conformation (see Cooper, 1980 for

review). Even in crystalline form when protein structures are subject to packing constraints, a variety of conformations may be exhibited and domains of proteins can move relative to each other, as described for insulin (for review see Chothia and Lesk, 1985). In the insulin molecule α-helices move by up to 1.3 angstroms with almost no significant change in backbone conformation. Since the extent of movement is limited, this can cause transmission, and possibly amplification, of conformational changes within the protein molecule.

A number of reports describe antibody-induced conformational changes in viral glycoproteins that appear to be transmitted, because topographically distinct regions of the molecule are modified. Lubeck and Gerhard (1982) showed that binding of monoclonal antibodies to one site on the HA is enhanced by simultaneous binding of antibody to a different epitope. Also using monoclonal antibodies, Heinz et al. (1984) mapped domains on the structural glycoprotein of tick-borne encephalitis virus to which binding is enhanced by antibodies binding to other sites. In addition, Fab fragments derived from these antibodies showed the same relationships, demonstrating that bivalent binding is not required to induce conformational changes in this protein. Sindbis virus has two envelope proteins, E1 and E2, and these are found in infected cells as a complex of E1 with a precursor of E2 protein (E1-PE2) which is stable in non-ionic detergent. Two monoclonal antibodies to E1 mutually enhance binding and also cause the dissociation of E1-PE2 complexes (Clegg et al., 1983). With the same monoclonal antibodies, enhancement of binding to Sindbis virus infected cells occurs (Chanas et al., 1982). Antibody-induced allosteric modification of the G protein of VSV has also been reported (LeFrancois and

Lyles, 1982).

The above provide indirect evidence for induction and transmission of conformational changes in proteins and direct evidence comes from the three-dimensional structure of the NA-NC41 Fab complex (Colman et al., 1987). NC41 Fab inhibits neuraminidase activity but does not sterically prevent access of trisaccharide substrate to the active site. An arginine residue at position 371, which normally points directly into the active site, is displaced slightly in the complex and this may cause the loss of activity. Monoclonal antibodies (four) to measles virus haemagglutinin decrease the intracellular expression of viral phosphoprotein, fusion and membrane protein (Fujinami et al., 1984). This antibody-induced antigenic modulation demonstrates that antibody to a determinant on the outside of the cytoplasmic membrane can initiate a transmembrane signal which modifies some structure on the cytoplasmic side.

Antibody-induced conformational changes in protein antigens that lead to modification of enzyme activity were reviewed by Celada et al. (1983).

(d) Conformational changes induced in virions by antibody

Influenza virus, adenovirus, rabbitpox virus and visna virus penetrate cells after neutralisation but fail to initiate infection. Neutralised influenza virus and visna virus uncoat and rabbitpox virus is partially uncoated. This implies that binding of antibody to a virion envelope protein results in modification of an internal component and for influenza virus it has been postulated that the HA transmits the signal (Possee et al.,

1982). There is evidence for antibody-induced conformational changes in the virions of picornaviruses. When bovine enterovirus is iodinated only one capsid protein VP1 is labelled, but after neutralisation with polyclonal antibody both VP1 and VP4 can be labelled indicating that a rearrangement of the surface components has been induced (Carthew, 1976). Studies with poliovirus have linked neutralisation with a change in the pI of virions. Infectious virus has a pI of approximately 7 and after neutralisation Mandel (1976) found the virus stabilised in a conformation with a pI of 4.5. Neutralising monoclonal antibodies also induce this change (Emini et al., 1983a,b; Icenogle et al., 1983) and papain digestion of virus-antibody complexes reverses the change and restores infectivity (Emini et al., 1983b). However, Emini et al. (1983a,b) noted that a neutralising polyclonal antibody to a single site on VP3 failed to induce the change in pI and infectivity was not reactivated by papain. Neutralising antibodies that fail to change the pI were also reported by Brioen et al. (1985). Additionally these workers showed that there was no quantitative relationship between neutralisation and change in pI where this occurred, and question Mandel's hypothesis. The situation remains to be resolved. It may not be realistic to expect neutralising antibody to cause gross changes in virion structure, detectable as changes in pI for example, particularly in less rigidly structured virions such as that of influenza virus.

Antibodies. 1. Monoclonal antibodies.

HC2 and HC61 monoclonal IgGs were purified from ascitic fluid provided by A.R.Douglas and J.J.Skehel, National Institute for Medical Research, London. These IgGs have HI and neutralising activity against H7 strains.

112/10/2R6 (called 2R6 herein). Ascitic fluid was produced in Balb/c mice using hybridoma cells provided by W.Gerhard (Wistar Institute, Philadelphia). The IgG purified inhibited NA activity of PR8 virus and an H7N1 reassortant used in this thesis, but not FPV/R (H7N1).

2. Polyclonal antibodies. Rabbit antisera were a gift from Dr. H.P.Taylor (Warwick).

Anti-H7N2 serum (WR69) was raised by inoculating 5 x 10^4 HAU of a reassortant virus in PBS into the ear vein on day zero and day 18. IgG was purified from serum obtained on day 25 i.e. 7 days after the second inoculation (B+7). This antibody has both HI and neutralising activity against FPV/R and the H7N1 reassortant used.

Antibody to bromelain-treated FPV/R (WR59) was used as a non-neutralising control. Bromelain-treated FPV/R (H7N1) (5 x 10^4 HAU) in PBS was inoculated into the ear vein on day zero and 23 days later. IgG was purified from serum obtained on day 34 i.e. 11 days after the second inoculation (B+11). This antibody lacked HI and neutralising activity but bound to H7 virus in a radioimmunoassay. (^{125}I)-labelled antibody co-pelleted with A/FPV/R but not B/Lee when incubated with virus and centrifuged through a 10% sucrose cushion.

3. Rabbit anti-mouse IgG (Fab specific) (RAM-Fab). Nordic Immunological Laboratories Ltd., Maidenhead, UK.

Cells. BHK-21 cells were a gift from Dr. J.Murphy (Warwick) and L-cells were provided by Dr. M.A.McCrae (Warwick).

Chemicals. m^7GpppAm - PL Biochemicals Ltd. Ribonucleoside triphosphates, ApG and melittin - Sigma, Poole, Dorset, UK. NP40 - BDH Chemicals Ltd., Poole, UK. Scintillation fluid - Beckman R2C, High Wycombe, UK. Radiolabelled compounds - Amersham International plc, Amersham, UK..

Eletrophoresis components. Specially pure acrylamide and bis-acrylamide - BDH. Nucleic acid markers - Oligo dT ladder, Bethesda Research Laboratories (BRL), Cambridge UK.

Enzymes. RNase A and T1 - Sigma. Bacterial alkaline phosphatase - Amersham. T4 polynucleotide kinase - BRL.

Tissue culture media and supports. Flow Laboratories, Irvine, Scotland. Serum - Gibco-BRL Ltd., Paisley, UK.

Viruses. Partially purified CPV was a gift from Dr. C.C.Payne, GCRI, Littlehampton, UK. Reovirus type 3 seed-stock was a gift from Dr. M.A.McCrae.

METHODS AND OPTIMISATIONS

1. CELLS

Primary chick embryo fibroblasts were prepared by the method of Morser et al. (1973). Plastic Petri dishes (5 cm diameter) were seeded with either 9×10^6 or 3×10^6 cells/dish in 3 ml of medium and incubated at 37°C until confluent. Roller bottles were seeded with 2×10^8 cells in 200 ml of medium to be confluent the following day.

BHK-21 cells were propagated in Glasgow Modified Eagle's Medium containing 5% NCS, glutamine (4 mM) and penicillin (100 units/ml) and streptomycin (100 µg/ml) (GMEM/NCS). 5 cm dishes seeded with 2.5×10^6 cells and 9 cm diameter dishes seeded with 5×10^6 cells were confluent the following day.

2. INFLUENZA VIRUS STRAINS AND THEIR PROPAGATION

Two influenza A viruses were used: A/FPV/Rostock/34 (H7N1)(FPV/R) and (FPV/R_M.PR/8_M), a reassortant virus selected from tissue culture medium produced by H.P.Taylor by coinfection of CEF with FPV/R and A/Puerto Rico/8/34 (H1N1)(A/PR/8) (Taylor and Dimmock, 1985). A small plaque was picked, plaque purified twice on CEF monolayers and then grown in eggs. This was neutralised efficiently by anti-H7 monoclonal and polyclonal antibodies and its neuraminidase activity was inhibited by a monoclonal anti-NA specific for the NA of PR/8. Hence, the designation of the reassortant as (FPV/R_M.PR/8_M).

Influenza viruses were grown in eggs by inoculating approximately 10^5 plaque forming units (pfu) into the allantoic cavity of

11-day embryonated hen's eggs and incubation for 20 hours at 37°C (FPV/R) or 36 hours at 33°C (reassortant). Eggs were chilled at 4°C for 4-16 hours and the allantoic fluid harvested and clarified by low-speed centrifugation. Virus was purified from allantoic fluid as described below or aliquots were snap frozen and stored at -70°C as virus stock.

3. PURIFICATION OF EGG-GROWN INFLUENZA VIRUS

The procedure described by Kelly and Dimmock (1974) was followed and all procedures were at 4°C. Clarified allantoic fluid was centrifuged at 75,000g for 90 min. The resulting pellets were soaked under PBS overnight, resuspended and clarified at low speed. The supernatant was loaded onto a 50 ml 15-45% (w/v) linear sucrose gradient in 10 mM Tris, pH 7.4 and 150 mM NaCl (TN) and spun at 60,000g for 90 min. The visible virus band was collected and diluted to 25 ml with PBS and loaded onto a 30-70% (w/v) linear sucrose gradient in TN and centrifuged for 16 hours at 60,000g. The virus band was collected, diluted to 45 ml with PBS and centrifuged at 75,000g for 90 min. The pellet was soaked overnight in PBS, resuspended and stored as aliquots at -70°C.

4. PREPARATION OF RADIOLABELLED INFLUENZA VIRUSES

(a)[^{35}S]-methionine-labelled virus

CEF monolayers in roller bottles were washed with PBS at 37°C and inoculated with 0.1 pfu of virus/cell in 10 ml per bottle of GMEM containing 1% NCS and 1/10th normal methionine (0.1x methionine) concentration. After adsorption for 1 hour at 37°C, 40 ml of the same medium was added with 1mCi of [^{35}S]-methionine and

incubation continued for 24 hours. Note that the cells were incubated in 0.1x methionine medium from inoculation as it was found that incubation in this medium for 24 hours prior to inoculation (Davey, 1984; Taylor, 1986) resulted in lower yields of virus with the same specific radioactivity (Table 3).

Virus was purified by the method of Dimmock et al. (1977) but unlabelled carrier virus was not added. All procedures were at 4°C unless indicated otherwise. Cell debris was removed from tissue culture medium by centrifugation at 1,000g for 10 minutes. Tris-HCl, pH 7.6 was added (final concentration 20 mM) to buffer the medium and $(NH_4)_2SO_4$ added, to give a 60% saturated solution, with stirring on ice. The precipitate was collected by centrifugation at 20,000g for 20 min, resuspended in 8 ml of PBS, and loaded onto a 55 ml 15-45% linear sucrose gradient. After centrifugation at 90,000g for 90 min, the gradient was fractionated into 2 ml aliquots and assayed for radioactivity and haemagglutinin. The fractions where the peaks of these activities coincided were pooled and diluted to 45 ml in PBS. The virus was pelleted by centrifugation at 75,000g for 90 minutes, resuspended in PBS, aliquoted and stored at -70°C.

(b) [^{32}P]-labelled virus

Confluent CEF monolayers in roller bottles were incubated for 24 hours in phosphate-free GMEM (GMEM-P)/1% NCS. The cells were washed in PBS and 10 ml of GMEM-P/0.1% NCS containing 1 pfu/cell added to each bottle. After incubation for 60 minutes at 37°C a further 40 ml of medium and 5 mCi of [^{32}P]-orthophosphate were added. Following incubation for 24 hours, virus was purified by a method similar to that described for [^{35}S]-methionine labelled

Table 3: Comparison of labelling regimes for production of [^{35}S]-methionine virus

Incubation in low methionine medium (cpm/HAU x 10^{-4})	Purified virus		Specific radioactivity
	HAU/ml (x10^{-8})	TCA-insoluble radioactivity (cpm x 10^{-8})	
From 24 h prior to inoculation	2.2	4.1	1.9
From inoculation only	20.0	41.1	2.1

Reassortant virus was grown in roller bottles in the presence of [^{35}S]-methionine. Both roller bottles were seeded with 2 x 10^{8} CEF cells and incubated overnight at 37°C. The medium of one was changed to low methionine GMEM with 1% NCS and the other 199/1% NCS. After a further 24 h incubation both roller bottles were inoculated and incubated as described in the text.

virus. The protocol varied in that the concentration of NCS was adjusted to 1% before $(NH_4)_2SO_4$ precipitation to provide carrier protein and virus was concentrated from sucrose gradient fractions by a second $(NH_4)_2SO_4$ precipitation in an endeavour to maintain infectivity. The second $(NH_4)_2SO_4$ precipitate was resuspended in 1 ml of PBS, dialysed overnight against PBS and stored in aliquots at -70°C.

5. PURIFICATION OF IgG FROM ASCITIC FLUID OR SERUM BY PROTEIN A-SEPHAROSE CHROMATOGRAPHY

Ascitic fluid or serum was diluted 1/2 in binding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) and passed 5 times through a column of protein A-sepharose in the same buffer. The column was washed with binding buffer until the absorbance at 280 nm (A_{280}) of the eluate was less than 0.1. IgG was eluted with pH 3.0 buffer (150 mM NaCl adjusted to pH 3.0 with acetic acid). Fractions of eluate were collected and the A_{280} measured. IgG-containing fractions were pooled and the pH adjusted to 7.0 by addition of 0.1 M NaOH. The concentration of IgG was determined (1 A_{280} unit = 1.34 mg/ml) and the IgG aliquoted and stored at -70°C. Columns were readjusted to pH 8.0 by washing in binding buffer and stored at 4°C.

6. PRODUCTION OF Fab FRAGMENTS FROM IgG

A modification (A.S.Carver, personal communication) of the method of Hudson and Hay (1980) was followed.

IgG (5-10 mg/ml) was incubated with pepsin (1 mg pepsin/25 mg of IgG) for 16 hours at 37°C in 70 mM sodium acetate-acetic acid,

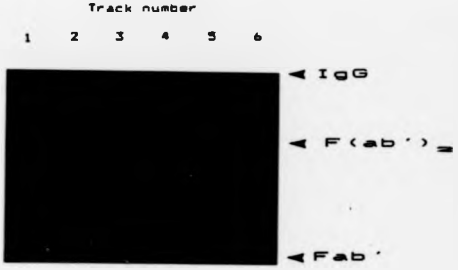


Figure 17: PAGE analysis of Fab', $F(ab')_2$ and IgG

HC61 (anti-H7) monoclonal IgG and fragments were analysed under non-reducing conditions and stained with coomassie brilliant blue.

Key:

1.	IgG	40 µg
2.	$F(ab')_2$	40 µg
3.	$F(ab')_2$	12 µg
4.	$F(ab')_2$	4 µg
5.	Fab preparation	25 µg
6.	Fab preparation	50 µg

The quantity of $F(ab')_2$ in the Fab preparation was judged to be 12/50 or 1/4 (compare tracks 3 and 6).

pH 4.0. (Stock buffer prepared by mixing 18 ml 0.2 M sodium acetate and 0.2 M acetic acid). The solution was adjusted to pH 8.0 by addition of NaOH (1 M) and passed through a protein A-sepharose column to remove Fc and undigested IgG. Cysteine (20 mM) was added to reduce $F(ab')_2$ and the mixture incubated at 37°C for 2 hours. The protein concentration was measured (1 A_{280} unit=1.34 mg/ml), cytochrome c added to 0.5 mg/ml and the solution dialysed for 16 hours against 1/10 PBS at 4°C to remove cysteine. The Fab preparation was aliquoted, lyophilised and stored at -70°C. Prior to use aliquots were resuspended in 1/10 original volume of water.

Figure 17 shows the products of one Fab preparation. $F(ab')_2$ were still present (approximately 1/4 of the total protein, judged by eye). $F(ab')_2$ may be present because of reassociation of Fab fragments after removal of cysteine or because reduction was incomplete. (Reassociation can be prevented by alkylation with iodoacetamide (Hudson and Hay, 1980)).

7. NEUTRALISATION ASSAYS

Residual infectivity of antibody-treated virus was assayed by plaque titration in CEF cells. Dilutions of virus-antibody mixtures (100 µl) were inoculated onto confluent monolayers, incubated at room temperature for 1 hour and overlayed with 199 medium containing 0.9% agar, 1% NCS and antibiotics. Plates were incubated at 37°C for 2-3 days and plaques stained with neutral red in PBS for 2-3 hours at 37°C.

Transcriptase activity and uncoating experiments were carried out using BHK-21 cells. However, the neutralisation titre was

Table 4: Neutralisation titre of FPV/R in CEF or BHK-21 cells

virus	CEF	BHK-21[a]
Non-neutralised virus (pfu/ml)[b]	1.1×10^{10}	3.5×10^{9}
Neutralised virus (pfu/ml)[c]	5.6×10^{7}	4.1×10^{7}
Neutralisation titre (%)	99.47	98.83

a. Infectivity titrations were carried out as described for CEF cells except that the overlay was GMEM containing 0.9% agar and antibiotics but no NCS, and the plates were incubated for 5 days at 37°C.

b. Non-neutralising antibody was non-specific monoclonal IgG (185/1).

c. Neutralising antibody was anti-H7 monoclonal IgG (HC2).

essentially the same when assayed in CEF and BHK-21 cells, though BHK-21 cells apparently provide a less sensitive assay of infectivity (Table 4). Plaques developed faster using CEF cells: FPV/R plaques were visible in 2-3 days and (FPV/R,PR/8) virus plaques were visible after 3-5 days. Plaques took approximately 2 days longer to appear using BHK-21 cells and CEF cells were therefore more convenient.

8. DETERMINATION OF TCA-INSOLUBLE RADIOACTIVITY

Whatman No.1 filter paper discs (25 mm diameter) were spotted with samples (maximum volume 50 µl) and immersed in 10% TCA held on ice (10 ml per filter). Filters were washed four times for 10 minutes with 10% TCA on ice, twice for 1 minute with ethanol and air dried. Filter-bound radioactivity was determined by scintillation counting. In some determinations the second wash was carried out at 100°C to hydrolyse RNA.

9. INFLUENZA VIRUS TRANSCRIPTION IN VITRO

(a) Assay for elongation activity

The conditions used were based on assays for elongation activity using ApG (Plotch and Krug, 1977), mRNA (Bouloy et al., 1978) and cap analogues (Penn and Mahy, 1984) as primers. Reaction conditions are given in Table 5. The mixtures were made up on ice, virus being added last, and transcription started by raising the temperature to 30°C. To monitor reactions, aliquots were assayed for TCA-insoluble radioactivity.

The optimum concentration of melittin used to activate

Table 5: Composition of reaction mixtures to assay influenza virus transcription *in vitro*

Basic reaction mixture:

	Final conc.
ATP	0.4 mM
CTP	0.4 mM
GTP	0.4 mM
[^{3}H]-UTP	1.5 µM (75 µCi/ml)
Tris-HCl, pH 8.0	50 mM
KCl	150 mM
$Mg(CH_3.COO)_2$	5 mM
DTT	5 mM

To these were added: virus

primers: mRNA

ApG

cap analogue

in the quantities shown in figure legends.

To activate transcription the virion membrane was disrupted with either NP40 (0.25%) or melittin 100 µg/ml (see text for details of optimisation)

transcription instead of detergent was 100 µg/ml for both (FPV/R.PR/8) virus in the absence of antibody (Figure 18) and FPV/R in the presence of non-specific IgG (Figure 19). This is the same as the optimum for VSV (Witt et al., 1981) and avian retroviruses (Boone and Skalka, 1980). Witt et al. (1981) noted the narrow range of concentration over which melittin permeabilised VSV, but when a narrower range of melittin was titrated 100 µg/ml was still optimum for activation of influenza virus (Figure 20). Boone and Skalka (1980) state that transcriptase activity of influenza virus was 3-fold higher when activated by melittin than by NP40. When NP40 and melittin were compared, transcriptase activity was always higher when NP40 was used (Table 6), suggesting that the concentration of NP40 used by Boone and Skalka (1980) was sub-optimal.

The RNase inhibitor bentonite improved the incorporation of radioactivity into macromolecules (Figure 21) and was included in reactions containing NP40. Melittin did not activate transcription in the presence of bentonite.

(b) Assays for initiation of influenza virus transcription

Reaction conditions were similar to those described for elongation assays containing NP40 and mRNA primer, but the only ribonucleoside triphosphate included was [^{32}P]-GTP (Plotch et al., 1981) at a concentration of 20 µCi per 50 µl reaction (1 µM). At least 2.5 x 10^3 HAU of virus per reaction were required to give detectable quantities of radiolabelled initiation products, as shown in Figure 22(a). A time-course demonstrated that the quantity of radiolabelled product increased over a period of 2 hours (Figure 22(b)) but all subsequent

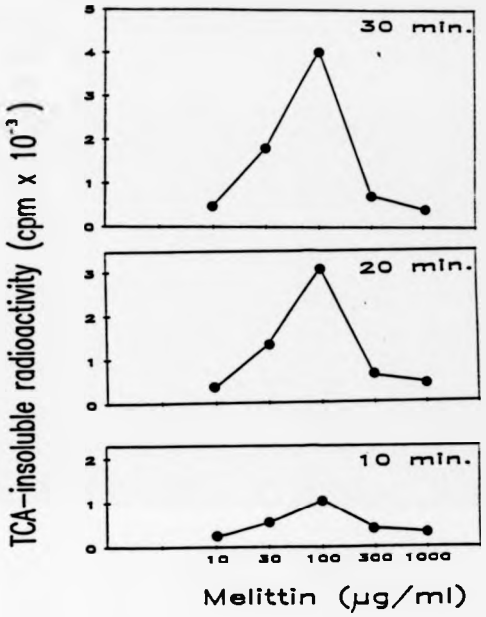


Figure 18: Transcriptase activity of (FPV/R,PR/B) in the presence of melittin (10-1000 µg/ml)

Transcriptase reactions (200 µl) contained 3 x 10^{8} HAU of (FPV/R,PR/B), 0.4 mM ApG, but no bentonite. Melittin was included at the concentration shown. The TCA-insoluble radioactivity in 15 µl samples was determined and the means plotted.

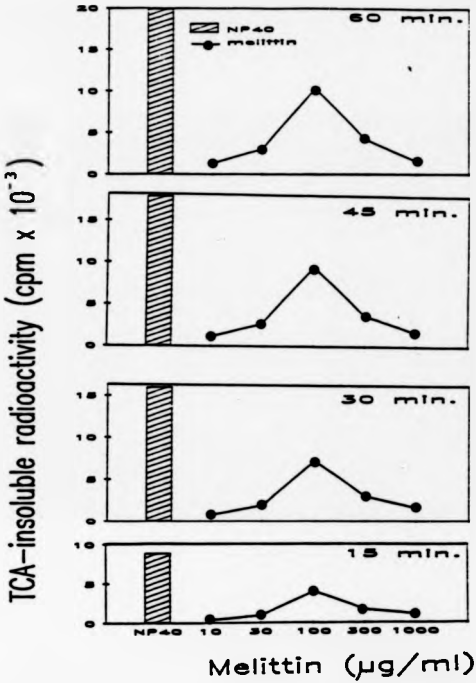


Figure 19: Transcriptase activity of (FPV/R.PR/8) in the presence of melittin (10-1000 µg/ml) or NP40 (0.25%)

Transcriptase reactions (200 µl) contained 3.5 x 10^{8} HAU of FPV/R, 0.4 mM ApG, 100 µg of IgG from a pre-immune rabbit antiserum (WR17), but no bentonite. Either melittin, at the concentration shown, or NP40 (0.25%) was used to activate transcription. The mean values for the TCA-insoluble radioactivity in duplicate 15 µl samples are shown.

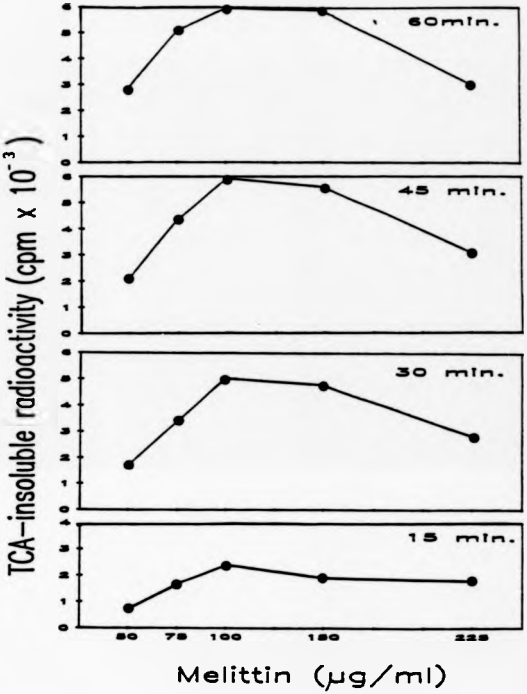


Figure 20: Transcriptase activity of FPV/R in the presence of melittin (50-225µg/ml)

Conditions as for Figure 19, but no results for NP40 disrupted virus are shown.

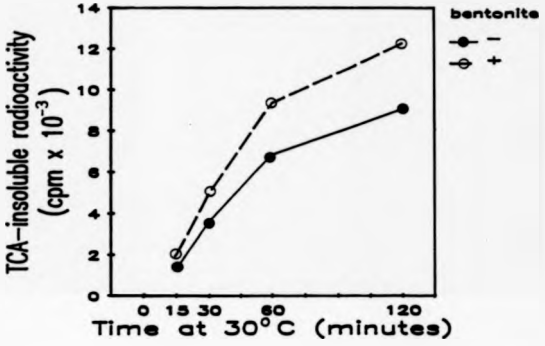


Figure 21: Effect of bentonite on incorporation of radioactivity into macromolecules

Transcriptase reactions (200 µl) contained 2.5 x 10^{3} HAU (FPV/R.PR/8), 7.5 µg of CPV mRNA, NP40 and no RNase inhibitor (●) or bentonite at 100 µg/ml (○). The TCA-insoluble radioactivity in duplicate 15 µl samples was determined and the mean values plotted.

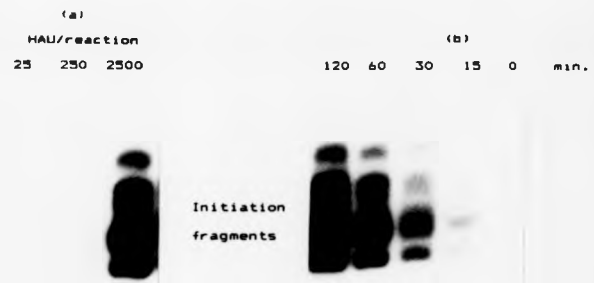


Figure 22: PAGE analysis of initiation fragments

See Results and Discussion Section I for a discussion of the size of initiation fragments.

(a) Initiation reactions containing the quantities of (FPV/R.PR/8) shown were incubated at 30°C for 60 minutes.

(b) Initiation reactions containing 2.5×10^{3} HAU of (FPV/R.PR/8) were incubated at 30°C for the times shown.

Table 6: Comparison of transcriptase activity of influenza virus activated with NP40 or melittin

Expt.	TCA-insoluble radioactivity (cpm x 10^{-3})		NP40 / melittin
	NP40	melittin	
A	20.0	10.5	1.9
B	15.0	8.0	1.9
C	16.5	6.0	2.8

Results for Experiment A are shown in Figure 19. All experiments included FPV/R, non-specific IgG and either NP40 (0.25%) or melittin (100 µg/ml).

reactions were incubated for 60 minutes.

At the end of the reaction 50 µl of 20 mM Tris, pH 8.0, 300 mM LiCl, 2 mM EDTA (2× TLE)/0.2% SDS was added with [^{3}H]-labelled RNA to act as a marker for the recovery and 5 µg of yeast tRNA to act as carrier in the following purification. Unincorporated [^{32}P]-GTP was removed by spun column chromatography (Maniatis et al., 1982) using Sephadex G25 in 1× TLE/0.1% SDS. The eluate was phenol extracted and ethanol precipitated and the quantity of TCA-precipitable tritium radioactivity was determined. Equal quantities were loaded onto slab gels of 16% acrylamide/0.8% bisacrylamide (Sanger and Coulson, 1978) and electrophoresed until the bromophenol blue marker had migrated 21 cm. Centrifugation of the reaction products through a single Sephadex G25 column was sufficient to remove unincorporated radiolabel, but ethanol precipitation removed little of the unincorporated [^{32}P]-GTP (Figure 23).

10. GROWTH AND PURIFICATION OF REOVIRUSES

Reovirus type 3 (Dearing Strain) (reovirus 3) was grown in L-cells in suspension culture as detailed by McCrae (1985).

Occluded cytoplasmic polyhedrosis virus type 1 (CPV) particles were purified using a modification (Mertens, 1979) of the method described by Miura et al. (1968). Partially purified polyhedra were washed four times in deionised, distilled water (ddH_2O) and collected by centrifugation at 4,000g. The final pellet was resuspended in 0.2 M Na_2CO_3-$NaHCO_3$ buffer, pH 10.8 and incubated at room temperature for 5 minutes. The suspension was diluted 1/3 with ddH_2O, debris pelleted at 4,000g, and the supernatant

Figure 23: Page analysis of initiation reaction products: effect of purification by spun-column chromatography

An initiation reaction mixture (250 µl), equivalent to 5 standard reactions, contained 13 x 10^3 HAU of (FPV/R.PR/8), 35 µg of CPV mRNA, 200 µCi of [α-^{32}P]-GTP, NP40 and bentonite. After incubation for 1 hour at 30°C 50 µl samples were purified as follows.

1. Diluted to 100 µl with 2x TLE/0.2% SDS, passed through 2 Sephadex G25 columns, phenol extracted and ethanol precipitated.

2. As 1, but passed through a single Sephadex G25 column.

3. Diluted to 100 µl with 2x TLE/0.2% SDS, phenol extracted, ethanol precipitated, resuspended and reprecipitated with ethanol.

4. As 3, but precipitated only once with ethanol.

layered onto a 12 ml 10-50% linear sucrose gradient and centrifuged at 70,000g, for 2 hours at 4°C. The visible virus band was collected, diluted 1 in 3, and pelleted by centrifugation at 85,000g for 2 hours at 4°C. The pelleted virus particles were resuspended after soaking overnight in a small volume of ddH_2O and the concentration estimated from the absorbance at 260 nm.

11. TRANSCRIPTION OF REOVIRUS mRNA IN VITRO

Reovirus 3 mRNA was transcribed under conditions described by Furuichi and Shatkin (1976) and detailed by McCrae (1985) which produce mRNA with capped and methylated 5' termini.

CPV mRNA was synthesized by the method of Smith and Furuichi (1980). Figure 24 shows a comparison of the incorporation of [^{3}H]-UTP in two reaction mixtures, both lacking the RNase inhibitor bentonite (Table 7). RNA synthesis was only appreciable in the presence of proteinase K. CPV is stable to this enzyme, but ribonucleases are destroyed and therefore lack of detectable mRNA synthesis in the absence of this enzyme was attributed to RNase contamination. In all subsequent reactions the reaction mixture described by Smith and Furuichi (1980) was used i.e. as Table 7(i) but including bentonite at 100 µg/ml.

12. EXTRACTION AND PURIFICATION OF RNA

The concentration of RNA samples was calculated from the absorbance at 260 nm (A_{260}) using the equation 1 A_{260} unit = 50 µg/ml. Purity of samples was judged by calculating the ratio

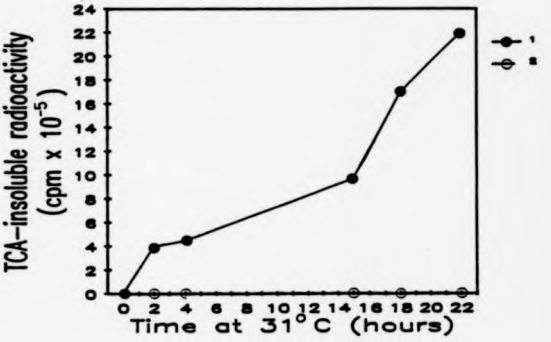


Figure 24: Comparison of two reaction mixtures for mRNA synthesis using CPV

RNA polymerase reactions (100 µl) contained 25 µg of CPV particles. 10 µl samples were assayed for incorporation of [^{3}H]-UTP into macromolecules by TCA precipitation.

Conditions as given in:

1. Fig. 4 of Smith and Furuichi (1980) but lacking bentonite.

2. Mertens and Payne (1983) but lacking bentonite.

See Table 7.

Table 7: Composition of mRNA synthesis reaction mixture for CPV

	1		2	
ATP	4	mM	2	mM
CTP	2	mM	2	mM
GTP	2	mM	2	mM
UTP	0.5	mM	0.5	mM
[^{3}H]-UTP*	1	µM	1	µM
AdoMet	1	mM	0.5	mM
Tris-HCl, pH 8	70	mM	60	mM
$Na(CH_3.COO)$	100	mM	-	
$Mg(CH_3.COO)_2$	10	mM	-	
Proteinase K	100	µg/ml	-	
CPV	250	µg/ml	250	µg/ml

1. Conditions as given in Fig.4 of Smith and Furuichi (1980), but lacking bentonite.
2. Conditions as Mertens and Payne (1978), but lacking bentonite.

* 1 µM [^{3}H]-UTP = 50 µCi/ml

See Figure 24.

of A_{260} and absorbance at 280 nm (A_{280}). Pure RNA has an A_{260}/A_{280} ratio of greater than 2.0. RNA was stored frozen or under ethanol at -20°C. All containers used were either made of glass and baked at 180°C for at least 6 hours to inactivate ribonucleases, or sterile plastic.

(a) vRNA from purified influenza virus

Purified influenza virus was diluted to 1 x 10^8 HAU/ml with TLE and made 1% for SDS. To this was added an equal volume of a 1:1 mixture of redistilled phenol and chloroform saturated with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 1 mM EDTA (phenol/chloroform). The mixture was vortexed and the phases separated by centrifugation at 13,000g for 30 seconds in a microcentrifuge. The upper aqueous phase was removed and the interface reextracted with TLE/1% SDS. Aqueous phases were pooled and reextracted twice with phenol/chloroform. Phenol and chloroform were removed from the aqueous phase by extraction with ether. After three extractions, the majority of the ether was removed and residual ether evaporated by passing a stream of air over the surface. RNA was collected by ethanol precipitation: LiCl to 200 mM and 2.5 volumes of absolute ethanol were added and the resulting mixture incubated on dry ice for 5 minutes or at -20°C for at least 30 minutes. Precipitates were pelleted by centrifugation at 13,000g for 10 min at 4°C, washed with 80% ethanol and repelleted twice then dried under vacuum.

(b) Extraction of RNA from influenza initiation reaction mixtures

After initiation reaction mixtures had been passed through Sephadex G25 columns, the concentration of SDS was increased to

1% and the RNA extracted as described for vRNA.

(c) mRNA from reovirus RNA polymerase reaction mixtures

Reovirus 3 or CPV particles were removed by centrifugation. The supernatant was extracted once with water-saturated phenol, three times with ether and ethanol precipitated. Pellets were resuspended in TLE/0.1% SDS and purified by two rounds of chromatography on Sephadex G50 (Results and Discussion Section I, Figures 34 and 35). Columns were calibrated using blue dextran to identify the void volume. Reovirus mRNAs had an $A_{260/280}$ ratio between 2.05 and 2.2.

(d) Extraction of RNA from BHK-21 cells

The guanidinium/CsCl method described by Maniatis et al. (1982) was used. BHK-21 cells were scraped from tissue culture dishes into cold PBS(1.5 ml/dish) using rubber policemen. The cells were pelleted at 13,000g for 10 seconds and resuspended in 4 M guanidinium isothyocyanate stock (1 ml/10^7 cells) (4 M guanidinium isothyocyanate; 5 mM sodium citrate, pH 7.0; 0.1 M β-mercaptoethanol; 0.5% sarkosyl). The mixture was layered onto a 3.5 ml cushion of 5.7 M CsCl, 0.1 mM EDTA and centrifuged at 110,000g for 16 hours at 20°C. The RNA pellets, and therefore the supernatant was aspirated and the pellets resuspended by washing the tube bottoms twice with 10 mM Tris-HCl, pH 7.6, 200 mM LiCl, 5 mM EDTA and 1% SDS. The RNA solution was extracted once with a 4:1 mixture of chloroform and butanol, the aqueous layer removed, and the organic phase reextracted with aqueous buffer. Aqueous phases were pooled and precipitated with ethanol. The mean $A_{260/280}$ ratio was 1.6 +/- 0.1.

13. RADIOLABELLING OF INFLUENZA VIRION RNA FOR USE AS A HYBRIDISATION PROBE

5' OH groups suitable for labelling with polynucleotide kinase were created by partial alkaline hydrolysis of virion RNA (2.5 µg) in 100 mM $NaCO_3$ at 50°C for 1 hour. The reaction was terminated by cooling the RNA solution and adding Tris-HCl, pH 7.6 (final concentration 20 mM). The vRNA fragments were ethanol precipitated, resuspended and 5' end-labelled using polynucleotide kinase and [γ^{32}P]-ATP (see Table 8). Macromolecular constituents were separated by spun column chromatography, ethanol precipitated and resuspended in water.

The 5' ends of influenza virion RNA have the structure 5'pppGUA... (Young and Content, 1971) and two methods of creating 5' OH groups were compared, namely partial alkaline hydrolysis and dephosphorylation using bacterial alkaline phosphatase. Figure 25 shows the incorporation of radioactive label into vRNA prepared by these methods and their ability to hybridise to the products of an in vitro influenza virus transcriptase reaction. More radioactivity (3.5-fold) was incorporated into alkali-digested vRNA and this gave a stronger signal by dot-blot hybridisation than dephosphorylated vRNA.

14. DOT-BLOT HYBRIDISATION ANALYSIS OF RNA FROM INFECTED CELLS

RNA extracted from BHK-21 cells was denatured by the method of White and Bancroft (1982), applied to nitrocellulose (NTC) membranes held in a BRL dot-blot manifold and hybridised by a

Table 8: Composition* of reaction mixture for 5' end-labelling nucleic acid with polynucleotide kinase (forward reaction)

(a) 4x reaction buffer:

	Concentration in 4x buffer
Tris-HCl, pH 7.6	240 mM
DTT	60 mM
$MgCl_2$	40 mM
ATP	1.32 μM

4x reaction buffer was made up on ice and stored at -20°C.

(b) polynucleotide kinase reaction

	Volume (μl)
vRNA (2.5 μg, alkali digested) in H_2O	10
4x reaction buffer	6
[γ^{32}P]-ATP 20μCi	2
T4 polynucleotide kinase (2.5 units/μl)	2
H_2O	4

Incubation: 30 minutes, 37°C.

*from BRL product guide.

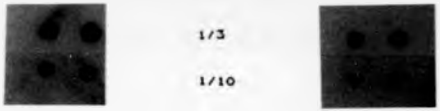
Figure 25: Comparison of 2 protocols for exposing 5' OH groups prior to radiolabelling vRNA for use as hybridisation probe

(a) Incorporation of radioactivity

Treatment of vRNA prior to radiolabelling	Total radioactivity incorporated (cpm x 10^{-6})[a]
none	0.16
partial alkaline hydrolysis	12.20
BAP-treatment[b]	3.47

(b) Comparison of hybridisation efficiency:

Dilutions of transcriptase reaction products[c] applied to the membrane



Notes:

a. Radioactivity was determined in scintillation fluid

b. Bacterial alkaline phosphatase (BAP) treatment was carried out as described by Maniatis et al. (1982) using 5 units of enzyme (Amersham) at 65°C for 1 hour in a volume of 100 µl.

c. The products of an in vitro transcriptase reaction were phenol extracted, ethanol precipitated, denatured (50% formamide, 10 minutes, 65°C) and applied to nitrocellulose.

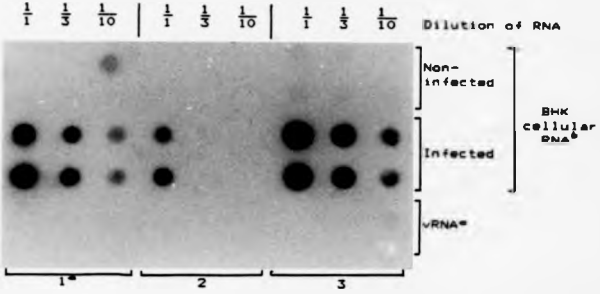
method based on Maniatis et al. (1982).

The NTC membrane was washed in water, then in 20x SSC and secured in a manifold connected to a vacuum pump. RNA was denatured in 50% formamide and 6% formaldehyde incubated at 55°C for 15 minutes. This method was found to be the best of three tested (Figure 26). After denaturation, the RNA was cooled on ice an equal volume of 20x SSC added, and the samples applied to the membrane. The filter was baked for 2 hours under vacuum at 80°C and transferred to hybridisation buffer (0.2 ml of buffer/cm^2 of membrane) without probe. The composition of hybridisation buffer is given in Table 9. The hybridisation buffer was degassed under vacuum before use and added to the filter contained in a bag. Care was taken to exclude air before sealing the bag. The filter was incubated at 42°C for 24 hours and then 3/4 of the buffer removed. The RNA probe was boiled in water (1/20 of the retained volume of hybridisation buffer) for 2 minutes, cooled and added to the hybridisation buffer. Hybridisation was carried out for 24-36 hours at 42°C. Filters were washed three times for 5 minutes at room temperature in 2x SSC and three times for 20 minutes in 0.25x SSC at 65°C. After air-drying, filters were autoradiographed at -70°C.

Transcripts (RNA+) within cells were detectable after 3 hours (Figure 27). Cells were also inoculated with various amounts of virus. (Figure 28). A multiplicity of infection (moi) of 10 was sufficient to give detectable levels of transcripts after 4 hours and increasing the moi from 10 to 1,000 gave only a 2-fold increase in the radioactivity bound to the filter.

Figure 26: Comparison of methods for denaturing RNA

DOT BLOT



QUANTITATION

Denaturation method	Mean radioactivity bound to infected cell RNA (1/1 dilu.)(cpm x 10^{-3})	Radioactivity relative to formamide-denatured samples
1	41.3	2.4
2	17.3	1.0
3	105.7	6.1

a. Denaturation methods: 1. 5 min at 100°C (RNA in H_2O); 2. 50% formamide, 65°C for 10 min; 3. 50% formamide/ 6% formaldehyde 55°C, 15 min.

b. Ratio of specific (infected cell) to non-specific (non-infected cell) hybridisation was 105651/3025=35.

c. 45 µg of cellular RNA at 1/1 dilution and 0.8 µg of influenza vRNA (single quantity at position marked 1/10).

Table 9: Composition of hybridisation buffer*

	Final concentration	
Formamide	50	%v/v
SSC	5	x
Denhardt's Reagent	1	x
Calf thymus DNA	250	µg/ml
EDTA	10	mM
SDS	0.5	%w/v

50x Denhardt's Reagent		20x SSC	
	g/100 ml		g/100 ml
Ficoll	1	NaCl	17.53
PVP	1	Sodium citrate	2.76
BSA	1	pH 7.0 with NaOH	

* from Maniatis et al. (1982).

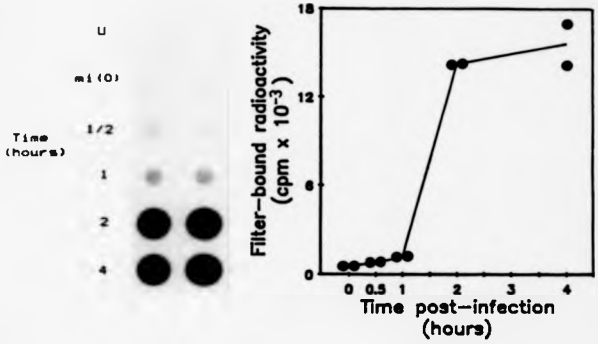


Figure 27: Time-course of appearance of influenza virus positive-sense RNA in BHK-21 cells

BHK-21 cells were inoculated with (FPV/R.PR/8) (moi=100) and incubated for the times indicated. The RNA was extracted and denatured with 50% formamide/6% formaldehyde at 55°C for 15 minutes. 18 µg of RNA was applied per duplicate. The graph shows the filter-bound radioactivity. Key: U=non-infected, mi=mock-inoculated (zero time) samples.

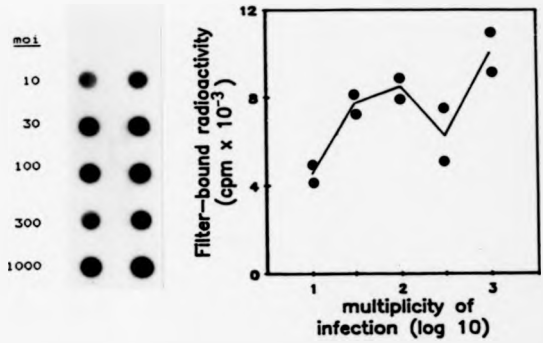


Figure 28: Dot blot hybridisation analysis of BHK-21 cells inoculated with various amounts of virus

BHK-21 cells were inoculated with (FPV/R.PR/8) at the moi shown and incubated for 4 hours. The RNA was extracted and denatured with 50% formamide/6% formaldehyde for 15 minutes at 55°C. 9 µg of RNA was applied per duplicate. The graph shows the amount of radioactivity bound to individual dots.

15. INHIBITION OF PROTEIN SYNTHESIS IN NON-INFECTED AND INFECTED BHK-21 CELLS WITH CYCLOHEXIMIDE

Protein synthesis was inhibited to prevent secondary transcription. Cells were inoculated in PBS and incubated in GMEM/NCS containing cycloheximide at 200 µg/ml (Avery and Dimmock, 1975). This concentration inhibited protein synthesis by nearly 99% (Table 10).

16. TRANSLATION OF mRNA IN VITRO

Nuclease-treated, message-dependent rabbit reticulocyte lysate (Amersham) was added to lyophilised [^{35}S]-methionine (80 µl lysate/0.5 mCi [^{35}S]-methionine). Reaction mixtures, made up on ice, contained 8 µl of radiolabel-containing lysate and 2 µl of mRNA (approximately 0.1 µg) and were incubated at 30°C for 60 minutes. Reaction mixtures were diluted in 10 mM Tris-HCl, pH 7.6, and samples assayed for TCA-insoluble radioactivity or analysed by PAGE.

17. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF PROTEINS

The gel systems used were based on that of Cook et al. (1979), containing 0.1% sodium dodecyl sulphate (SDS), and the Laemmli (1970) discontinuous buffer system. A stacking gel of 4.5% acrylamide/0.12% bis-acrylamide was used.

Antibodies and antibody fragments were analysed using a resolving gel of 7.5% polyacrylamide/0.2% bis-acrylamide. Samples were prepared by boiling for 2 min in 4% SDS, 6% glycerol, 10 mM Tris-HCl, pH 7.6. After electrophoresis at 14 mA for 16 hours,

Table 10: Inhibition of protein synthesis in BHK-21 cells by cycloheximide (CYH)

Cells	TCA-insoluble radioactivity (cpm) -CYH	+CYH	Inhibition of protein synthesis (%)
Infected	53960	589	98.9
Uninfected	82659	1119	98.7

BHK-21 cells were inoculated with FPV/R (moi=100) in PBS or mock-inoculated with PBS on ice for 1 hour then incubated in tissue culture medium at 37°C. CYH was included in these media at 200 µg/ml as indicated. After 3.5 hours at 37°C, [^{35}S]-methionine was added (20 µCi/ml) and incubation continued for a further 30 minutes. The cells were scraped into cold PBS (1.5 ml), sonicated, and samples (50 µl) assayed for TCA-insoluble radioactivity.

unlabelled proteins were detected by silver or Coomassie Brilliant Blue staining.

Silver staining was carried out exactly as detailed by Wray et al. (1981) after washing gels for at least 16 hours with 3 or 4 changes in 50% methanol.

Coomassie Brilliant Blue stain solution contained 0.5 g of Coomassie Brilliant Blue dissolved in 49 ml of methanol to which 49 ml of water and 18.2 ml of acetic acid was added. Gels were immersed in stain for 30-60 min at room temperature, destained in 50% methanol/7% acetic acid and dried under suction.

In vitro translation products and proteins from purified virions or infected cells were resolved on 10-30% linear polyacrylamide gradient gels. The ratio of acrylamide to bis-acrylamide was 212:1 and the gels contained 0-8% glycerol. Samples were reduced by boiling for 2 minutes in 5% 2-mercaptoethanol, 4% SDS, 6% glycerol, 10 mM Tris-HCl, pH 7.6. Electrophoresis was carried out at 14 mA for 16 hours. Gels were fixed in 50% methanol/7% acetic acid and immersed in Amplify (Amersham) for 20 minutes at room temperature. After drying onto filter paper under suction, gels were fluorographed at -70°C using Fuji Rx X-ray film.

18. ASSAY FOR RIBONUCLEASE SENSITIVITY OF INFLUENZA VIRION RNA

A method based on that of Koff and Knight (1979) was followed. Confluent BHK-21 cell monolayers (5 cm dishes) were inoculated with [^{32}P]-labelled influenza virus in PBS for 90 minutes on ice and incubated in PBS or tissue culture medium as described in figure legends. Monolayers were then washed with cold PBS,

scraped into 1.5 ml of cold PBS and pelleted by centrifugation at 13 000g, 10 sec. The pellets were resuspended in PBS (225 µl) and stored at -70°C.

To assay for ribonuclease (RNase) sensitivity, the cells were thawed and sonicated (2x 5seconds). Duplicate samples (50 µl) were assayed for TCA-insoluble radioactivity (untreated samples) and 100 µl of cell lysate added to 100 µl of RNase solution containing 100 µg of RNase A and 500 units of RNase T1. RNase digestions were incubated at 37°C for 30 minutes and then 4 samples (40 µl) were spotted onto filter paper discs. One pair of these filters was processed for TCA-insoluble radioactivity under standard conditions (RNase treated samples) and the other pair TCA-precipitated with boiling (boiled samples). Boiling in TCA hydrolyses RNA and the radioactivity which remains is in protein. The fraction of RNA sensitive to RNase was calculated using the following equation:

$$100 - [(R-B)/(U-B) \times 100] = \text{RNase-sensitive virion RNA (\%)}$$

Where: U = untreated sample (cpm/assay);
R = RNase treated sample (cpm/assay);
B = boiled sample (cpm/assay).

cpm/assay=(mean cpm/filter x 2) for untreated samples and (mean cpm/filter x 5) for RNase-treated and boiled samples.

Methods for disrupting cells were compared and the concentration of RNase enzymes titrated.

Figure 29 shows that maximal RNase sensitivity was seen with all disruption methods tried. It was concluded that one cycle of

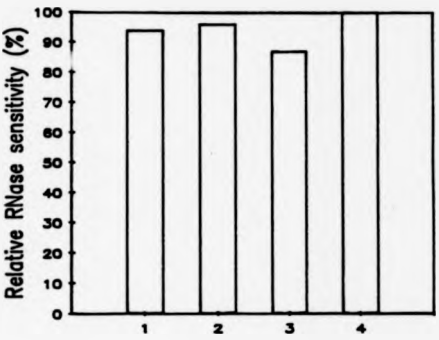


Figure 29: RNase sensitivity of BHK-21 cellular RNA after disruption of cells by freeze/thawing with and without sonication

BHK-21 cells (1×10^6 cells/5 cm dish) were incubated for 24 hours in GMEM/NCS containing [^{3}H]-uridine (1 µCi/ml) and 24 hours in the same medium without radiolabel. Cells from duplicate plates were disrupted according to the following key:

1. Freeze/thaw x1
2. Freeze/thaw x3
3. Freeze/thaw x1 and sonicated for 5s
4. As 3, but sonicated for 2 x 5 seconds

RNase sensitivity was assayed as described in the text except that the relative RNase sensitivity was calculated taking the maximum value as 100% (sample 4)

freeze/thawing was sufficient to allow access of RNase enzymes to the cell, but treatment with ultrasound was incorporated into the protocol as it aided pipetting of cell lysates.

Figure 30 demonstrates that the concentration of RNases used in assays of vRNA sensitivity was 10-fold in excess of that required to give maximal digestion of cellular RNA.

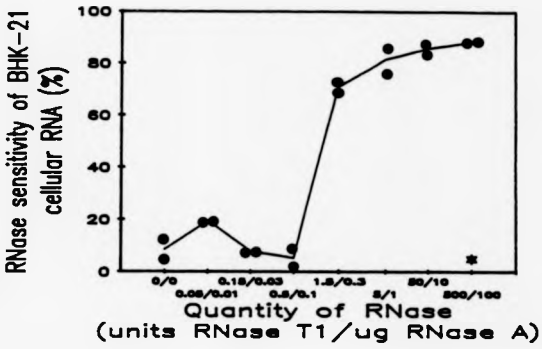


Figure 30: Titration of RNase concentration

BHK-21 cells were labelled with [^{3}H]-uridine as described in Figure 29. RNase sensitivity was assayed as described in the text but using the concentrations of RNase enzymes shown. * denotes the quantity of enzymes used in assays for RNase sensitivity of virion RNA and in Figure 29.

RESULTS
AND
DISCUSSION

SECTION I. Production of mRNA primer using reoviruses

1. INTRODUCTION

Capped and methylated mRNA was required as primer for influenza transcriptase assays and was produced using two members of the Reoviridae namely Bombyx mori (type 1) cytoplasmic polyhedrosis virus (CPV) and reovirus type 3, Dearing strain (reovirus 3). The double stranded RNA genomes of these viruses are transcribed in vitro into mRNAs by virion RNA dependent RNA polymerases. As shown here, and described by others, these enzymes are remarkably stable and therefore are particularly suitable for production of mRNA.

This section describes the production, purification and partial characterisation of these mRNAs. The ability of the mRNAs to prime influenza virus transcription was assayed and the efficiency of priming is discussed.

2. RESULTS

(a) Production of mRNA using Reovirus 3

An RNA synthesizing reaction mixture that gives capped and methylated mRNA was used, (Furuichi and Shatkin, 1976). [^{3}H]-UTP was included to monitor the reaction and Figure 31 shows the incorporation of this radiolabel into macromolecules during a polymerase reaction. A plateau is reached when some component of the reaction becomes limiting. The reovirus 3 cores are still capable of synthesizing mRNA: they can be recovered by

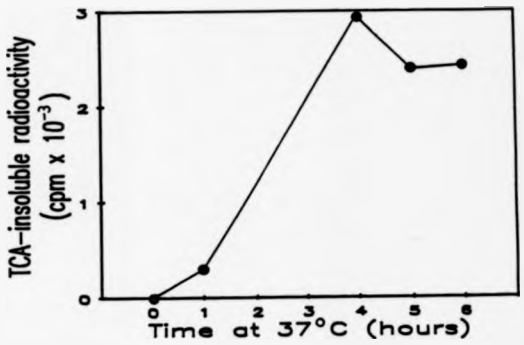


Figure 31: Incorporation of radioactive label into macromolecules during a reovirus 3 polymerase reaction

Reovirus polymerase reaction contained cores from 5 mg of virus and[^{3}H]-UTP. The filter precipitable radioactivity in 10 µl samples was measured.

centrifugation, resuspended in fresh polymerase buffer and will synthesize mRNA again. Thus one preparation of virus can be used in multiple rounds of mRNA synthesis (M.A.McCrae, personal communication). Figure 32 shows the results obtained when this procedure was followed. For the first two rounds, each of 6 hours duration, the final yields of filter precipitable radioactivity were similar, but in subsequent rounds little incorporation of label took place.

The level of incorporation of label at the end of round 1 is equivalent to 260 µg of mRNA, and 5 mg of reovirus 3 was used (52 µg mRNA/mg virus). Compared with published values this yield is poor. For example, Both et al. (1975), quote a yield of 500 µg of mRNA per mg of virus in a 60 minute reaction. Attempts were therefore made to synthesize mRNA using CPV.

(b) Production of mRNA using CPV.

The polymerase reaction mixture described by Smith and Furuichi (1980) was employed. This includes both proteinase K and bentonite as RNase inhibitors. My own initial experiments had shown the importance of including an RNase inhibitor (see Part II, Methods and Optimisations).

Yields of mRNA from a preparation of CPV were maximised in two ways: polymerase reactions were carried out for long periods of time and CPV particles were recycled in the way described for reovirus 3 above, and used in multiple rounds of mRNA synthesis. Figure 33 shows the incorporation of radiolabel during 5 rounds of mRNA synthesis and demonstrates the stability of the CPV polymerase. In total this batch of CPV was used in 7 reactions

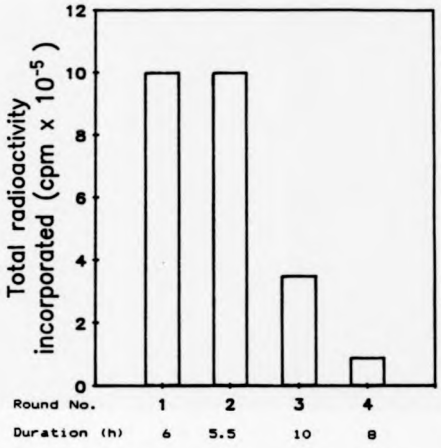


Figure 32: Total radioactivity incorporated at the end of four successive rounds of mRNA synthesis by reovirus 3

Conditions as Figure 31.

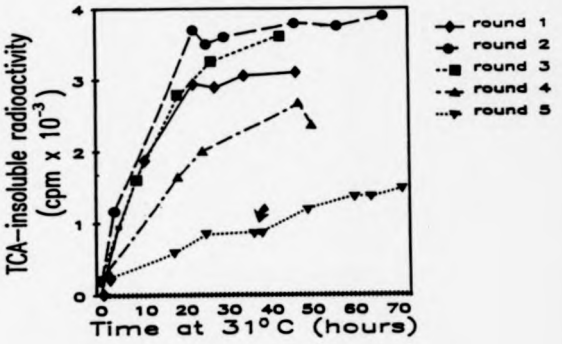


Figure 33: Multiple rounds of mRNA synthesis with CPV

The polymerase reaction initially contained 250 µg of CPV in 1 ml. R1 to R5 denote the successive rounds of synthesis. The arrow shows the point in round 5 at which fresh polymerase buffer was added (see text).

over a period of 16 days. The arrow on the curve for round 5 indicates that after 37 hours the volume of this reaction was doubled by addition of fresh polymerase buffer, allowing synthesis to begin again. This may be because the fresh reaction mixture provides a component that was exhausted or denatured after 37 hours incubation, or dilutes an inhibitory product below the critical concentration. The differences in the slopes of the linear portions of the curves are due, at least in part, to loss of CPV particles in samples withdrawn to monitor the reaction.

After purification of mRNA from reaction products the yield was determined individually for the first 3 rounds, see Table 11. Smith and Furuichi (1980) quote a yield of mRNA of 30 times input, i.e. genomic, RNA but do not give details of how this was calculated. 23-30% of the CPV particle is considered to be RNA, though 30% is thought to be the most accurate estimate (Payne and Mertens, 1983) and one strand acts as template. On this basis, the yield of mRNA at best was 8.4 times the input RNA. Whilst this suggests that yields could have have been increased, large quantities of mRNA were obtained.

(c) Purification of mRNA from reaction mixtures.

It was imperative that the mRNA be purified free of the components of the polymerase reaction. In particular ribonucleoside triphosphates (rNTPs) had to be removed. These would reduce the incorporation of radiolabelled rNTPs in assays of influenza virus elongation and assays of initiation activity, which depend on the incorporation of [^{32}P]-GTP only, would be impossible. Additionally, both CPV (Smith and Furuichi, 1982) and reovirus 3 (Yamakawa et al., 1981) synthesize a variety of

Table 11: Yield of purified CPV mRNA from 3 successive rounds of synthesis

Round of synthesis	Input CPV		Product	
	virus[a] (µg)	RNA[b] (µg)	mRNA (µg)	Product mRNA / Input CPV RNA
1	250	75	195	5.2
2	215	65	261	8.0
3	176	53	225	8.4

a. The amount of virus used in the first round was measured by determining the absorbance at 260 nm. The values for subsequent rounds was calculated by subtracting CPV lost during sampling and assuming 100% recovery by centrifugation between rounds.

b. Calculated by assuming that 30% of virus is RNA and a single strand acts as template

Results from experiment shown in Figure 33

oligonucleotides that potentially could interfere with priming of influenza virus transcription by mRNA (Table 12). The purification method employed was chosen to separate high molecular weight mRNA from rNTPs and oligonucleotides.

CPV mRNA was purified twice by gel filtration using Sephadex G50 which is capable of fractionating molecules of 1,500-30,000 molecular weight. Figure 34 is the elution profile from the first column and there are two peaks of radioactivity. The first elutes with the excluded volume (species of molecular weight greater than 30,000) and was assumed to be mRNA. The radioactivity in the second peak was TCA soluble. This contains unincorporated [^{3}H]-UTP and the other rNTPs. Oligonucleotides synthesized by CPV were analysed by Smith and Furuichi (1982) and the largest, m^{7}GpppApG, has a molecular weight of approximately 1,100. Therefore, all the oligonucleotides should elute with the rNTPs. When the mRNA was applied to a second column, Figure 35, a single peak of radioactivity was seen demonstrating that the mRNA eluted from the first column was free of [^{3}H]-UTP.

(d) Translation of mRNAs *in vitro*.

CPV and reovirus 3 mRNAs were translated in rabbit reticulocyte lysates to which [^{35}S]-methionine had been added. The products of these reactions were analysed by PAGE (Figure 36). Both reovirus 3 and CPV mRNAs directed the synthesis of polypeptides of discrete sizes. Tracks 1 and 2 show the profile of influenza virus and the molecular weights of the unglycosylated proteins are indicated. Using these as markers, and the information in Table 13, an estimate of the molecular weight of the CPV translation products in tracks 3 and 4 was made. This method of

Table 12: Oligonucleotides synthesized by CPV and reovirus 3 that could prime influenza virus transcription

virus	oligonucleotide	Proportion of *in vitro* transcription products
CPV	pppApG	22.7
	ppApG	
	GpppApG	1.0
	m^7GpppApG	
	GppppApG	1.0
reovirus 3	m^7GpppGU	3.0
	m^7GpppGCU	3.5
	m^7GpppGCUA	1.8

CPV data from Smith and Furuichi (1982).J.Biol.Chem. 257:485. Reovirus 3 data from Yamakawa *et al*. (1981). J.Biol.Chem. 256:6507.

m^7Gppp = 5' cap structure. The oligonucleotides shown are a subset of those formed in complete reaction mixtures by CPV or reovirus 3. Criteria for selection: All capped oligonucleotides would be expected to compete with capped mRNA for the cap-binding site of PB1 of the influenza virus transcriptase enzyme (Penn and Many, 1984); oligonucleotides including the sequence ...ApG would be expected to prime elongation (Kawakami *et al*., 1981b). See General Introduction Part 7(a).

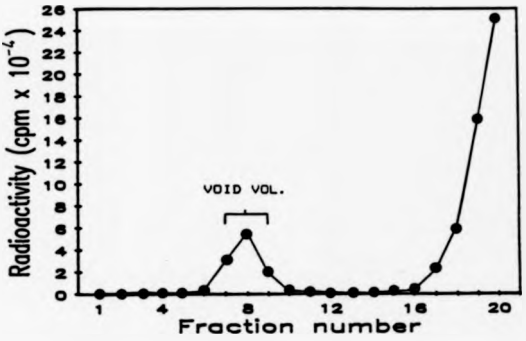


Figure 34: Separation of CPV polymerase reaction products by column chromatography

Polymerase reaction mixture was applied to a Sephadex G50 column (270 mm x 10 mm) in TLE (10 mM Tris, pH 8, 150 mM LiCl and 1 mM EDTA), 0.1% SDS. 800 µl fractions were collected and the radioactivity in 10 µl fractions determined. Fractions 7-11, corresponding to the void volume, were pooled and ethanol precipitated.

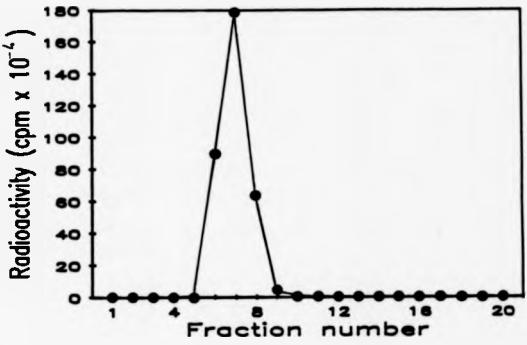


Figure 35: Elution profile of mRNA applied to a Sephadex G50 column

The pooled fractions from Figure 34 were resuspended in 200 µl of TLE and applied to a second Sephadex G50 (175 mm x 6 mm) column in the same buffer. 300 µl fractions were collected and the radioactivity in 10 µl samples determined. Fractions 6-9 were pooled and precipitated with ethanol.

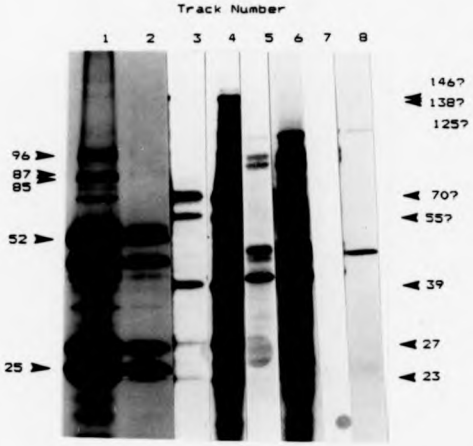


Figure 36: PAGE analysis of products of translation of CPV and reovirus 3 mRNAs in vitro

Key: Tracks

1 and 2. [^{35}S]-methionine labelled influenza virus

3. Translation products of CPV mRNA, 8.8 x 10^{4} cpm

4. As track 3 but 8.8 x 10^{5} cpm

5. Translation products of reovirus 3 mRNA, 8.7 x 10^{4} cpm

6. 72 hour exposure of track 5.

7. Endogenous polypepetides synthesized by rabbit reticulocte lysate.

8. 72 hour exposure of track 7.

16 hour exposures are shown, unless otherwise indicated.

Two exposures of the same track, or tracks loaded with different amounts of radioactivity, are presented to show proteins present in low abundance.

Table 13: Molecular weights of viral proteins and translation products of CPV

viral proteins[a] (mw x 10^{-3})	translation products[b] (mw x 10^{-3})
146	146
138	138
125	125
107	-
80	-
70	70
61	(61,58)
58	(58,55)
55	
39	(39)
31	-
27	27
23	23

a. From Fig. 14, Payne and Mertens (1983).

b. From Fig. 13, Payne and Mertens (1983). Translation products of CPV mRNA in rabbit reticulocyte lysate. Brackets denote estimated molecular weights of polypeptides not marked by the authors.

assessment is only approximate but suggests that polypeptides of the expected molecular weight were produced. None of the polypeptides from the reovirus 3 translation reactions, tracks 5 and 6, migrate with the molecular weights expected for reovirus 3 proteins (Both et al., 1975).

(e) Priming of influenza virus transcription by mRNA.

The mRNA produced in vitro using reovirus 3 and CPV should be a mixture of 10 species of mRNA. The WSN strain of influenza virus preferentially cleaves mRNAs at A residues 10-13 nucleotides from the 5' cap in the presence of GTP (Plotch et al., 1981). From the sequence of mRNAs it is therefore possible to predict which will act as primers. Consideration of the reovirus 3 sequences (Antczak et al., 1983) suggests that 6 of the mRNAs should be utilised, yielding initiation fragments of 3 sizes. Sequence information for CPV is less comprehensive, but 3 of the 5 published mRNA sequences contain an A residue within 10-13 nucleotides of the cap (Kuchino et al., 1982) and initiation fragments of at least two sizes would be expected. All the mRNAs synthesized were presumed to have methylated 5' cap structures and therefore be recognised by PB2, the cap binding protein. The mRNA molecules not capable of acting as primer will compete with primer molecules and this would be expected to limit the priming efficiency. Use of unfractionated reovirus mRNA has been reported (Bouloy et al., 1979). The experiments described below were designed to test the ability of the mixed populations of mRNAs to prime influenza transcription.

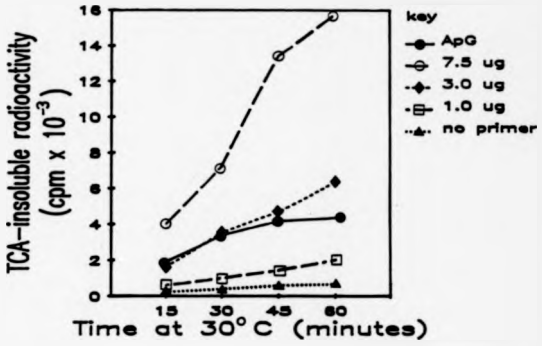


Figure 37: Stimulation of influenza virus transcription by CPV mRNA

Influenza virus elongation reactions (200 µl) contained 3.5 x 10^3 HAU of (FPV/R.PR/8) virus and the quantities of CPV mRNA shown or ApG at 0.4 mM. The TCA-precipitable radioactivity in 15 µl samples was determined.

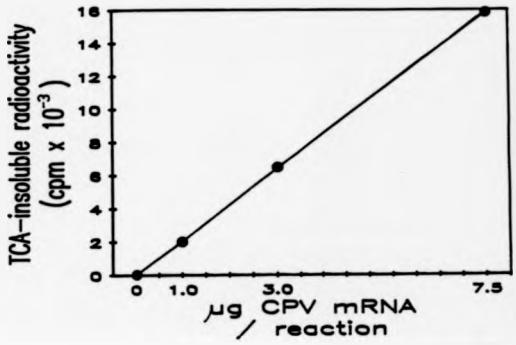


Figure 38: The relationship between transcriptase activity and the quantity of CPV mRNA primer added

The ordinate shows the radioactivity incorporated after incubation of transcriptase reactions at 30°C for 60 minutes. Values from Figure 37.

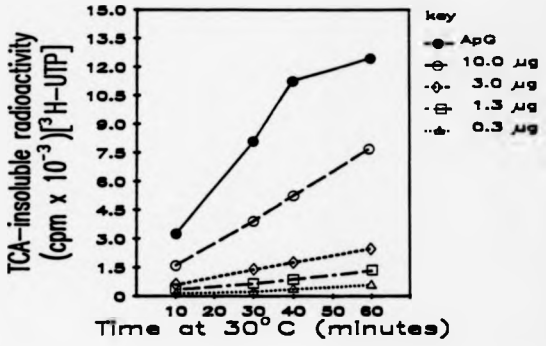


Figure 39: Stimulation of influenza virus transcription by reovirus 3 mRNA

Influenza virus elongation assays contained 3.5×10^3 HAU of FPV/R and the quantities of reovirus mRNA shown or ApG (0.4 mM final concentration). The TCA-precipitable radioactivity in 15 µl volumes was determined.

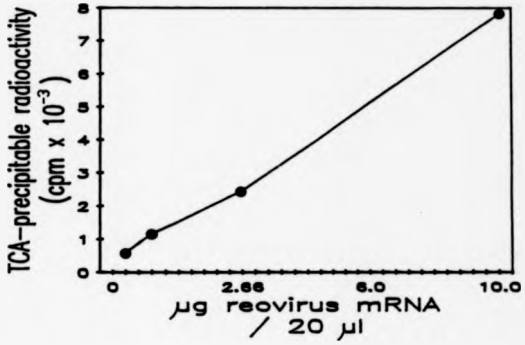


Figure 40: The relationship of transcriptase activity to the quantity of reovirus 3 mRNA primer added

The ordinate shows the radioactivity incorporated after incubation of transcriptase reactions at 30°C for 60 minutes. Values from Figure 39.

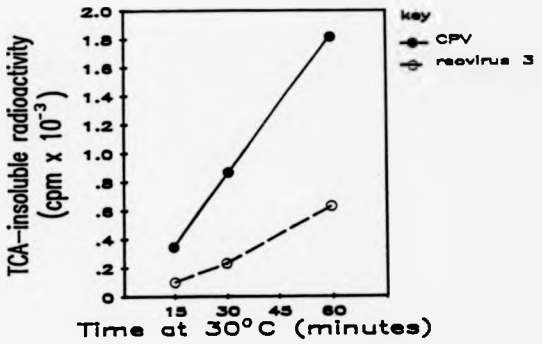


Figure 41: Direct comparison of the priming ability of CPV and reovirus 3 mRNA

Influenza virus transcriptase reactions (200 µl) contained 2.7×10^{3} HAU of (FPV/R.PR/8) virus and 1 µg of either CPV or reovirus mRNA. The ordinate shows the TCA-insoluble radioactivity in 15 µl volumes.

mRNA	Reaction rate (cpm incorporated/min)
CPV	32.5
reovirus 3	12.5

ratio CPV/reovirus 3 = 2.6

Table 14: Comparison of the molar concentrations of mRNAs and ApG primers required to give equivalent incorporation of radioactivity in influenza virus transcriptase assays

mRNA type	Ratio $\frac{[ApG]}{[mRNA]}$	Reference
CPV	9,500	Figure 37
reovirus 3	1,900	Figure 39
CPV	7,100	Kawakami and Ishihama (1983)
reovirus 3	1,000-2,000	Bouloy *et al.* (1979)

Sample Calculation

Values taken from Figure 37.

Primer	Concentration	Radioactivity incorporated (at 30 min)
CPV mRNA	9×10^{-8} M	7,233
ApG	4×10^{-5} M	3,389

Concentration of mRNA that would give the same incorporation of radiolabel as ApG $= (3,389/7,233) \times 9 \times 10^{-8}$

$= 4.2 \times 10^{-8}$ M

Ratio $[ApG]:[mRNA] = 4 \times 10^{-5} : 4.2 \times 10^{-8} = 9,500$

The value for the molecular weight of reovirus mRNA, 7×10^{6}, was derived from Bouloy *et al.* (1979). An average molecular weight for CPV mRNA was calculated from the values for CPV genomic RNA and their relative transcription frequencies (Payne and Mertens, 1983).

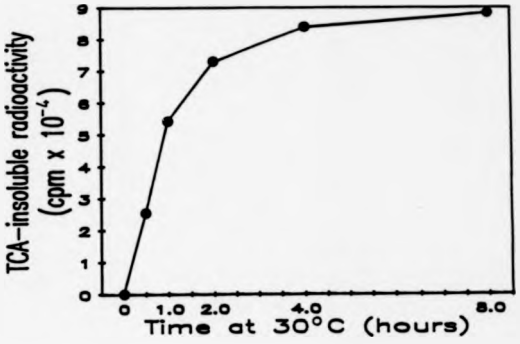


Figure 42: Messenger RNA-dependent transcriptase activity of FPV in the presence of CPV mRNA over 8 hours

The polymerase assay mixture (200 µl) contained 2.5×10^{3} HAU of FPV and 5 µg of CPV mRNA. The TCA-insoluble radioactivity in 15 µl samples was determined.

(i) Stimulation of influenza virus elongation reactions by mRNA.

CPV and reovirus 3 mRNAs were added to influenza virus elongation reactions. Both stimulated incorporation of radioactive label suggesting that they were functioning as primers; results obtained using CPV and reovirus 3 mRNA are shown in Figure 37 and Figure 39, respectively. The incorporation of radioactive label in these reactions was proportional to the quantity of mRNA added, over the range tested (Figure 38 and Figure 40).

A direct comparison was made of the ability of CPV and reovirus 3 mRNAs to stimulate elongation. Figure 41 shows that CPV mRNA is more efficient. The rate is 2.6 times faster for the CPV-primed reaction compared to that primed by reovirus 3 mRNA.

The polymerase reaction progressed at an approximately linear rate over 60 minutes in the presence of CPV mRNA (Figure 37 and 45). This raised the question of what proportion of the input virion RNA was being transcribed. Calculation 1 shows that after 2 hours mRNA equivalent to the input virion RNA had been transcribed. Incubation for periods of longer than 2 hours did not increase this proportion (Figure 42). Assuming that full-length transcripts were being produced, this suggests that reinitiation does not occur.

(ii) Priming activity of CPV mRNA produced in different rounds

The RNA polymerase activity of type 1 CPV is stimulated up to 60 times by S-adenosyl methionine (AdoMet), (Furuichi, 1974), but repeated pelleting of CPV during purification reduces this effect

Calculation 1: Estimate of the proportion of influenza virion RNA transcribed in a polymerase reaction primed with CPV mRNA

Influenza transcriptase reactions contained 2,500 HAU in 200 µl.

Since 10^{8} HAU is equivalent to 300 µg protein this represents 7.5 µg of protein/200 µl.

1% of influenza virus is RNA, therefore 7.5 x 10^{-8}g RNA/200 µl.

The molecular weight of the influenza virus genome is approximately 4 x 10^{6} therefore reactions contained:

$$7.5 \times 10^{-8} / 4 \times 10^{6} = 1.9 \times 10^{-14} \text{ moles/reaction}$$

The influenza virus genome consists of approximately 14,000 nucleotides. Assuming that 1/4 are A residues:

$$1.9 \times 10^{-14} \times (14{,}000/4) = 6.6 \times 10^{-11} \text{ moles of A residues/reaction}$$

Radioactivity ([^{3}H]-UTP) incorporated into macromolecules

= 5.6 x 10^{6} cpm/15 µl (Figure 43)

= 7.5 x 10^{8} cpm/reaction.

Since the counting efficiency of [^{3}H] on filters in the LKB counter used = 12% there were 6.3 x 10^{8} dpm/reaction.

The specific radioactivity of [^{3}H]-UTP was 9.3 x10^{14} dpm/mole.

Therefore:

no. of molecules of UMP incorporated

= 6.3 x 10^{8} / 9.3 x 10^{14}

= 6.7 x 10^{-11}

Product/substrate therefore = 6.7 x 10^{-11}/6.6 x 10^{-11} = 1, i.e. mRNA equivalent to the quantity of input RNA was transcribed.

(Payne and Mertens, 1983). AdoMet also acts as a methyl donor in the methylation of mRNA caps (Furuichi, 1974). Since CPV particles were pelleted between rounds of synthesis it was possible that the mRNAs produced might vary in the extent of methylation and this would affect the ability to prime influenza virus transcription because only cap 1 structures, $m^7GpppAm...$, are efficiently recognised by the PB2 protein of influenza virus (Bouloy et al., 1979). However, mRNA from different rounds of synthesis were equally competent in stimulating elongation (Figure 43). Having established this, the mRNAs from succesive rounds were pooled.

(iii) Priming of initiation reactions by mRNA.

CPV and reovirus 3 mRNAs were added to influenza virus initiation assays. Under these conditions capped mRNAs are recognised, cleaved at A residues 10-13 nucleotides from the cap producing primer fragments to which [^{32}P]-GTP is added at the 3' ends. The products include initiation fragments 12-15 nucleotides in length (10-13 nucleotides plus G residues at the cap and 3' end) and labelled with ^{32}P. These initiation fragments were analysed by PAGE under conditions capable of resolving short pieces of RNA differing in length by a single base. Multiple initiation fragments were expected from reactions primed by both reovirus 3 and CPV mRNA. In the reactions primed by reovirus mRNA only one size was produced (Figure 44). The CPV mRNA-primed reaction yielded initiation fragments of 3 sizes, with a fourth band visible on longer exposures.

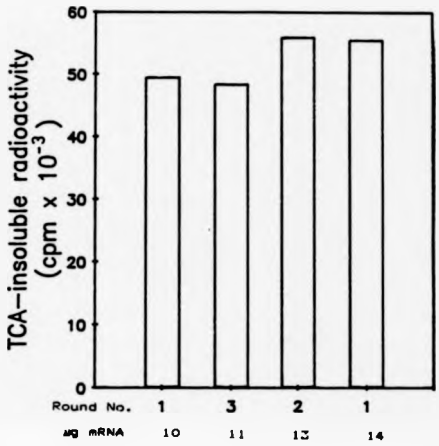


Figure 43: Comparison of priming activity of CPV mRNA from different rounds of synthesis

Transcriptase reaction mixtures (200 µl) contained 3.5×10^{8} HAU of (FPV/R.PR/8) and the quantities of CPV mRNA shown. The TCA-insoluble radioactivity in 15 µl samples was determined after incubation for 120 minutes at 30°C.

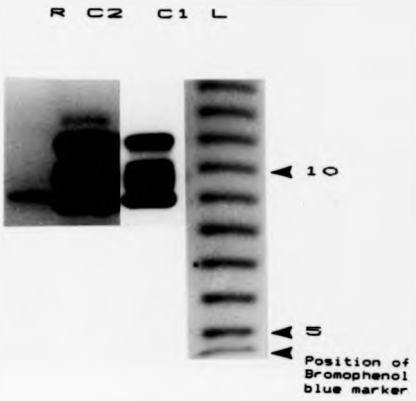


Figure 44: PAGE analysis of the products of initiation reactions primed with reovirus 3 and CPV mRNA

The RNA products of initiation reactions were separated on a 16% polyacrylamide gel. Reovirus 3 (R) and CPV (C) mRNAs were used to prime reactions. C2 is a longer exposure of C1 and L=oligo dT ladder.

3. DISCUSSION

Reovirus mRNAs were produced for use as primers to assay mRNA-dependent transcription of influenza virus *in vitro*.

Table 14 shows that the molar concentrations of CPV and reovirus 3 mRNAs required to give the same level of incorporation of radioactivity were 9,500 and 1,900 times lower respectively than ApG, in agreement with values published by Bouloy *et al.* (1979) and Kawakami and Ishihama (1983).

The products of initiation assays primed with reovirus 3 mRNA were not as expected: only a single size of initiation fragment was seen when 3 sizes of fragment were expected. The incomplete sequence data for CPV suggests that initiation fragments of 2 sizes should be produced, but in fact 2-4 bands were seen (Figs. 44 and 49 of Section II). This pattern would be produced if a single [^{32}P]-GMP moiety was added to primer fragments of discrete sizes but could also arise by extension of a single primer fragment by addition of multiple [^{32}P]-GMP moieties as described by Plotch *et al.* (1981). Since a mixed population of mRNA was used, there is no way of distinguishing between these two possibilites.

Attempts were made to determine the size of the initiation fragments by comparison with a series of oligo dT markers with a size range quoted as 4-22 nucleotides. If the fastest migrating species is 4 nucleotides long (see Fig. 44 and Fig. 49 of Results and Discussion Section II) then the initiation fragments migrated in the same position as species of 9, 10, 11 and 12 nucleotides. Initiation fragments of 12-15 nucleotides in length were anticipated. The shortfall of 3 nucleotides may be the result of one or a combination of the following. The fastest

migrating band of the oligo dT markers co-migrated with the buffer front and may therefore mark the position of molecules longer than 4 nucleotides. The oligo dT preparation may not contain the range of sizes specified; for example, some preparations contained more than 19 bands (Figure 49). The presence of the 5' cap in initiation fragments may affect mobility. Alternatively, it may be that FPV cleaves shorter primer fragments than WSN. A reduction of 3 bases would yield initiation fragments 11 and 12 nucleotides long and a difference of 4 bases would yield primer fragments of a single size (11 nucleotides) from reovirus 3 mRNA. Differences in transcriptase specificity between strains are unlikely since sequence information suggests the polymerase proteins are highly conserved (McCauley and Mahy, 1983; Robertson et al., 1984; Roditi and Robertson, 1984; Kemdirim et al., 1986).

The results obtained here show that CPV mRNA is a more efficient primer than reovirus 3 mRNA on a weight basis (Figure 41) and by comparison to ApG on a molar basis (Table 14). This mRNA preparation also gave proteins with molecular weights corresponding to CPV proteins when translated in vitro and was used in all subsequent experiments.

SECTION II: The effect of neutralisation on transcriptase activity *in vitro*

1. INTRODUCTION

Possee (1981) and Possee *et al.* (1982) reported that the transcriptase activity of two influenza viruses was inhibited by neutralising antibody. When FPV was treated with a polyclonal antibody specific for the HA, transcriptase activity was 7-fold lower than the control. The transcriptase activity of the X-49 reassortant was inhibited 9-fold by a monoclonal neutralising IgG. The quantities of antibody used reduced infectivity by 99.9% or more i.e. the inhibition of transcriptase was not proportional to neutralisation. Later Shimizu *et al.* (1985) studied the effects of a panel of 8 monoclonal IgGs specific for the HA of influenza virus A/Aichi/68. Only one of these neutralised infectivity and inhibited transcriptase activity (5.6-fold). Of the remainder, four neutralised infectivity but did not inhibit transcription and 3 had no effect on neutralisation or transcriptase activity. This suggests that the epitopes that mediate inhibition of transcription are a sub-set of the neutralisation determinants. However, mapping of the binding sites of these monoclonal antibodies has not been documented.

Influenza virus is unique in the mechanism used to cap mRNA. *In vivo* the virus removes caps from cellular mRNAs and these form the 5' ends of influenza virus mRNAs. This process can be mimicked *in vitro* and the precise mechanism involved has been elucidated (General Introduction, Part 7(a)). The transcriptase

enzyme binds to the 5' cap of heterologous mRNA and endonucleolytically cleaves the donor mRNA at an A residue 10-13 nucleotides from the cap. Initiation involves addition of the first base, invariably a G residue, and is followed by elongation (Braam et al., 1983). The product is influenza virus mRNA with a short heterologous sequence covalently attached to the 5' end. Cap analogues with the structure $m^7GpppNm$ stimulate transcriptase activity but are not incorporated into transcripts (Penn and Mahy, 1984; Kawakami et al., 1985). This suggests that capped mRNA stimulates transcriptase activity allosterically, as well as acting as primer, because PB2 is the cap-binding protein and PB1 carries out initiation and elongation (Braam et al., 1983).

The transcriptase assays reported by Possee et al. (1982) and Shimizu et al. (1985) were performed either in the absence of primer or using ApG. Under these conditions only elongation occurs (see General Introduction Part 7(a)). Functional mRNA is synthesized by influenza virus only in the presence of capped heterologous mRNA which acts as a source of ready-made 5' caps. Thus, if neutralising antibody had been predominantly inhibiting cap recognition, allosteric stimulation, or the endonuclease function, elongation would not have been an appropriate parameter to measure; inactivation of the cap-utilising functions could reduce infectivity without having a profound effect on elongation in vitro.

This section describes experiments designed to test the hypothesis that neutralisation inactivates the cap-utilising functions of influenza virus.

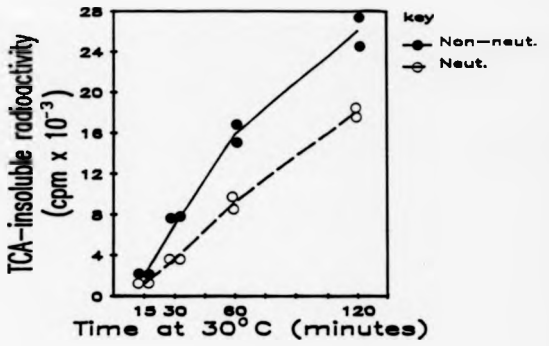
2. RESULTS

(a) Transcriptase activity of virus reacted with monoclonal antibody

The simplest means of assaying the cap-utilising functions is by mRNA-dependent elongation assays. These included CPV mRNA and all four ribonucleoside triphosphates, one of which being radiolabelled to allow the reaction to be monitored.

Figure 45 shows the transcriptase activity of virus after incubation with specific antibody. Virus was either neutralised by 99.99% with a monoclonal anti-HA IgG or incubated with anti-NA IgG as a control. The latter binds to the virus but does not neutralise infectivity. The kinetics of the transcriptase reaction are similar regardless of the antibody used to treat the virus. The neutralising antibody reduced transcriptase activity, but the maximum inhibition was less than 2-fold. This is not significant compared to the 5 to 9-fold inhibition seen by others (Possee et al., 1982; Shimizu et al., 1985).

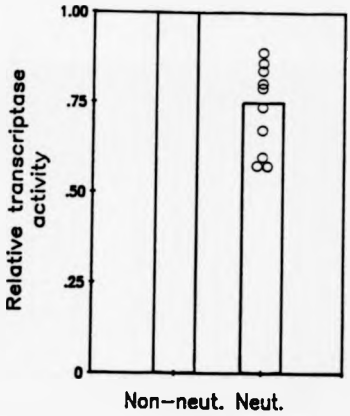
Figure 46 is a summary of 10 experiments using the HC2 and 2R6 monoclonal antibodies where the transcriptase activity of neutralised and non-neutralised virus were compared. This shows that the transcriptase activity of virus treated with neutralising antibody was reduced by 1.3-fold. For all the experiments neutralisation was 99.9% or more and it is concluded that this neutralising antibody does not significantly reduce transcriptase activity measured by mRNA-dependent elongation assays.



Inhibition (Non-N./Neut.)	1.7	1.9	1.7	1.4

Figure 45: Kinetics of transcriptase reactions of non-neutralised and neutralised virus in vitro

Each reaction mixture (200 µl) contained 3.5 x 10^3 HAU of (FPV/R.PR/8) virus (H7N1) which had been incubated with either anti-HA (HC2) or anti-NA (2R6) IgG (16 µg/10^3 HAU). The infectivity of neutralised virus (○) was 99.99% lower than that of non-neutralised virus (●). CPV mRNA (7.5 µg/reaction) was added as primer. Duplicate samples (15 µl) were withdrawn at the times shown and the TCA-insoluble radioactivity determined. Assays were performed in duplicate and the mean for duplicate assays is plotted.



IgG specificity:	anti-NA	anti-HA
Binding:	+	+
IgG clone:	2R6	HC2

Figure 46: Transcriptase activity of virus after incubation with monoclonal antibodies

Summary of the results of 10 experiments where transcriptase activity of (FPV/R.PR/8) virus was assayed in vitro after incubation with specific IgGs. Transcriptase reaction mixtures containing CPV mRNA were sampled after 60 minutes at 30°C and filter-precipitable radioactivity determined. The incorporation of radiolabel in reactions containing virus treated with anti-NA IgG was defined as 1 and the transcriptase activity of neutralised virus calculated relative to this. Values for individual experiments are denoted by ○. In every experiment virus treated with anti-HA was neutralised by 99.99% or more.

(b) Transcriptase activity of virus reacted with polyclonal antibody

Results of experiments using monoclonal antibodies may be of limited relevance because of the fine specificity of the paratope. Therefore, transcriptase activity was assayed after neutralisation with a polyclonal rabbit antiserum which would be expected to contain antibody to some, if not all, antigenic sites and many epitopes. (However, this assumption may not be valid-see Discussion, below).

Since anti-NP antibodies inhibit transcriptase activity (van Wyke et al., 1981) virus-antibody complexes were separated from free antibody by centrifugation before assaying transcriptase activity. This prevents antibodies to internal proteins which may be present from reacting after disruption of the virion with detergent. Transcriptase activity of neutralised virus was compared to virus reacted with non-neutralising anti-HA, raised against bromelain-treated virus, and IgG from pre-immune serum. Figure 47 shows that transcriptase activity was unaffected by polyclonal IgG despite the infectivity of the neutralised virus being reduced by 99.9%.

The results presented above show that there is no significant reduction in transcriptase activity after neutralisation. This is strong but indirect evidence that the cap-utilising functions are not affected by neutralisation. This investigation was continued by measuring allosteric stimulation, part (c), and utilisation of capped mRNA by analysis of the products of the initiation of transcription, part (d).

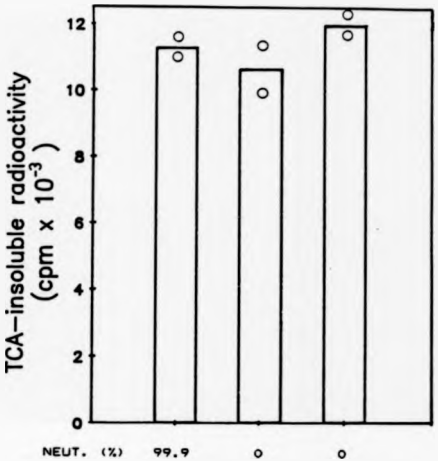


Figure 47: Transcriptase activity of virus after incubation with IgG from polyclonal antisera

(FPV/R.PR/8) virus was incubated with IgG (60 µg/10^{3} HAU) and virus-antibody complexes pelleted through 5% sucrose and resuspended. Transcriptase assays (100 µl) contained approximately 4 x 10^{3} HAU and 5 µg of CPV mRNA. The TCA-insoluble radioactivity in duplicate 10 µl samples (O) was determined after incubation for 30 minutes at 30°C.

(c) Allosteric stimulation of transcription by cap analogue $m^7GpppAm$

Allosteric stimulation of transcription was measured in elongation assays containing cap analogue $m^7GpppAm$. The results in Figure 48 show that the transcriptase activity of non-neutralised virus was stimulated 4-fold by this cap analogue. This is approximately the same as that reported by other authors (Penn and Mahy, 1984; Kawakami et al.,1985). The transcriptase activity of neutralised virus was also 4-fold higher in the presence of cap analogue. However, compared to the non-neutralised virus, the transcriptase activity of neutralised was reduced by 1.2-fold. Therefore neutralisation does not prevent allosteric stimulation nor does it result in a significant reduction in transcriptase activity.

(d) Assay of the initiation reaction of RNA synthesis

Initiation was assayed in reactions containing CPV mRNA but only one ribonucleoside triphosphate ([α ^{32}P]-GTP). Under these conditions cap binding, cleavage and initiation occur, but there is no elongation. The products of these reactions include primer fragments 11-14 nucleotides long with [^{32}P]-GMP incorporated at the 3' end. Figure 49 shows the RNA fragments produced in initiation assays by virus treated with monoclonal antibodies. Two sizes of initiation fragment are seen and the reasons for this, and sizing of these fragments has been discussed (see Discussion, below and Results and Discussion Section I). Regardless of which IgG was used to treat the virus, the heterologous mRNA was recognised, cleaved and used as primer,

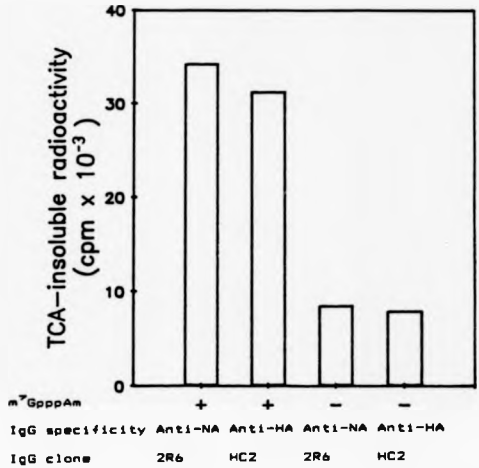


Figure 48: Transcriptase activity of antibody-treated virus in the presence and absence of cap analogue

Each reaction mixture (25 µl) contained 2.2 x 10^8 HAU of (FPV/R.PR/8) virus which had been incubated with the IgG shown (22 µg/10^8 HAU). Cap analogue $m^7GpppAm$ was included as shown (+). Duplicate 5 µl samples were withdrawn after 60 minutes at 30°C and the TCA-insoluble radioactivity determined. The values plotted are the means of duplicate assays. Virus incubated with anti-HA was neutralised by 99.99%.

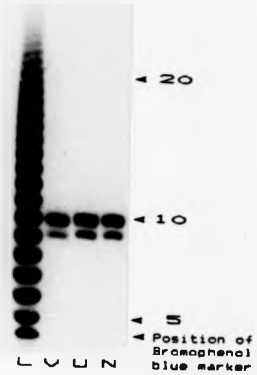


Figure 49: PAGE of the products of initiation assays of non-neutralised and neutralised virus

(FPV/R.PR/8) virus (2.5×10^8 HAU/assay) was incubated at 25°C for 1 hour with monoclonal anti-HA (HC2), anti-NA (2R6) (25 µg IgG/10^8 HAU) or PBS. Initiation assays (50 µl) contained 5 µg of CPV mRNA. After incubation for 1 hour at 30°C the RNA was extracted and analysed on a 16% polyacrylamide gel. The labelled products were detected by autoradiography at -70°C with an intensifying screen. N: neutralised virus (99.99% neutralisation); U: non-neutralised virus; V: virus incubated with PBS and L: 5' labelled oligo dT_{6-22} ladder (BRL); BPB marks the position of bromophenol blue dye.

apparently with the same efficiency as virus that had not been treated with antibody. This is direct evidence that the cap-utilising activities of neutralised virus are functional.

(e) Activation of transcriptase by melittin.

Before assaying transcriptase activity *in vitro* the virion has to be disrupted and commonly the non-ionic detergent NP40 is used. The precise effects of detergents vary depending on the amounts of virus and detergent used and with virus strain. Kawakami and Ishihama (1983) showed that NP40 solubilises the lipid membrane and also partially disrupts the protein structure of influenza virus so that HA, NA and M are dissociated from the RNP core. For antibody to the HA to effect neutralisation by inhibiting transcriptase activity (Possee *et al.*, 1982) it is necessary to hypothesise firstly that transmembrane communication mediated by viral proteins occurs and secondly that this is altered when virus is neutralised. It was of interest therefore to activate transcriptase activity of neutralised virus with an agent like melittin which would not be expected to interfere with protein-protein interactions. Melittin is a 26 amino-acid polypeptide present in bee venom that acts as an ionophore (Williams and Bell, 1972). It has been used to activate virion enzymes of VSV (Boone and Skalka, 1980; Witt *et al.*, 1981), an avian retrovirus and influenza virus (Boone and Skalka, 1980). Melittin associates as tetramers in membranes introducing pores that allow passage of solutes but does not solubilise membranes (Dawson *et al.*, 1978).

The optimum concentration of melittin was determined and transcriptase activity was 0.5-fold of that obtained using NP40

(see Methods and Optimisations, Part 9(a)). Melittin was used to assay transcriptase activity of virus treated with neutralising anti-HA IgG or anti-NA IgG. Figure 50 shows that transcriptase activity of neutralised virus was 1.4-fold lower than that for infectious virus. Again this reduction is insignificant compared to that seen by other workers and considering the reduction in infectivity.

3. DISCUSSION

The possibility that neutralisation of infectivity was associated with inhibition of some stage in the utilisation of heterologous mRNA by the transcriptase enzyme *in vitro* has been thoroughly investigated. All four stages, namely cap-binding, allosteric stimulation, endonucleolytic cleavage and initiation occurred after neutralisation. Additionally the kinetics of the transcriptase reaction of neutralised virus were similar to those of non-neutralised virus. Polyclonal and monoclonal neutralising antibodies were used but only with the monoclonal antibody was any inhibition of transcription apparent. However, this inhibition was small and judged to be insignificant compared to the 9-fold and 5.6-fold reported by Possee *et al.* (1982) and Shimizu *et al.* (1985) respectively. Unfortunately the antibodies used by Possee (1981) were no longer available as it would have been of interest to determine if the mRNA-primed transcription was inhibited to the same extent as elongation.

At least two types of neutralising epitope exist, those that mediate inhibition of transcription and those that do not (Shimizu *et al.*, 1985). The data presented here can be reconciled with those of Possee (1981) if it is assumed that the IgGs from

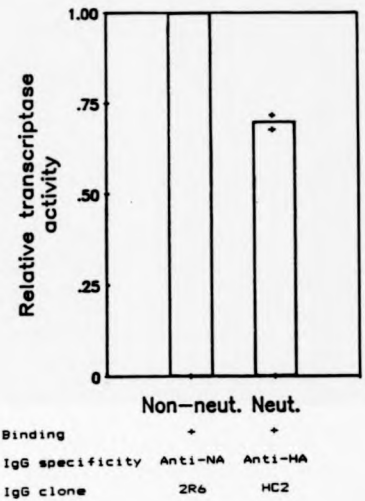


Figure 50: Transcriptase activity of virus incubated with specific monoclonal antibodies and activated with melittin

Transcriptase assays contained (FPV/R.PR/8) virus which had been incubated with the IgGs shown, melittin (100 µg/ml) and CPV mRNA. Relative transcriptase activity was calculated as in Figure 46. The infectivity of neutralised virus was reduced by 99.9%.

the polyclonal antiserum and the monoclonal IgG were directed to epitopes that do not mediate inhibition of transcription. This possibility is easy to accept for the monoclonal antibody given the precise specificity of these reagents and there is evidence that the paratopes present in polyclonal antisera are not of the broad specificity expected. Limited anti-HA responses have been documented for human (Natali et al., 1981; Wang et al., 1986) and animal sera (Laver et al., 1981). Natali et al. (1981) showed that human antisera are often unable to bind to antigenic variants selected with a single monoclonal antibody. These escape mutants usually only vary from the parent by a single amino-acid (Laver et al., 1980). Escape mutants have also been generated using a hyperimmune antiserum and these mutants showed an altered reactivity to only one of six monoclonal antibodies (Taylor and Dimmock, unpublished results). A truly polyclonal antiserum would not be expected to select escape mutants since the mutation rate is too low for mutants to be generated when selection is carried out using two monoclonal antibodies specific for different epitopes (Yewdell et al., 1979). Thus, the polyclonal antiserum used may lack the paratope capable of inhibiting transcription. Unfortunately this realisation came too late for me to assay the effects of other monoclonal and polyclonal antibodies on transcriptase activity.

Shimizu et al. (1985) provide clear evidence that neutralisation is not accompanied by inhibition of transcriptase activity *in vitro*. The effect of the monoclonal antibody that inhibited transcription (Shimizu et al., 1985) was reversed by incubating antibody treated-virus with higher concentrations of NP40. These authors conclude that the inhibition of transcriptase seen *in vitro* is the result of interference in the disruption of virus

with detergent and discuss the relevance of activation *in vitro* to uncoating *in vivo*. *In vivo*, sub-viral particles are liberated under conditions of low pH when the HA fuses the viral and endosomal membranes (Patterson and Oxford, 1986). Activation of transcription *in vitro* with NP40 may be harsher than uncoating *in vivo*, as suggested by Shimizu *et al*. (1985), or alternatively exposure of virus to low pH could be important. These possibilities could be investigated *in vitro* by exposing virus to low pH before activation with detergent or melittin, or uncoating virus by fusion in endosomes *in vitro* (Davey *et al*., 1986b) prior to assaying transcription.

SECTION III: Effect of neutralisation on transcription *in vivo*

1. INTRODUCTION

Influenza virus transcription *in vivo* can be divided into two stages on the basis of the requirement for protein synthesis. The first stage is primary transcription and occurs in the presence of cycloheximide, an inhibitor of protein synthesis. During primary transcription the input virus synthesizes mRNA using virion RNA as template. In cycloheximide-treated cells, cRNA synthesis is only 5-15% of that seen in controls (Mark *et al.*, 1979). If protein synthesis is not inhibited secondary transcription takes place: mRNA is synthesized in larger quantities and full-length cRNA is produced to serve as template for vRNA synthesis (General Introduction, Part 7).

Possee *et al.* (1982) showed that neutralised FPV/R did not give rise to detectable levels of RNA transcripts in CEF cells. RNA was extracted and analysed after 3 hours at 37°C, by which time secondary transcription would be occurring. This says that the replication of neutralised virus is interrupted prior to secondary transcription. The possibility that neutralised virus could direct primary transcription, producing quantities of mRNA which were undetectable by the method used, remained to be tested. Possee *et al.* (1982) detected cRNA synthesis by annealling the total RNA extracted from cells so that newly synthesized cRNA would protect the [^{32}P]-labelled RNA of the inoculum virus from RNase degradation. The amount of virion RNA in hybrid molecules which is resistant to ribonuclease digestion is thus a measure of transcription (Bean and Simpson, 1973;

Stephenson and Dimmock, 1975) but this method is not sensitive enough to detect primary transcription (Possee, 1981). In the experiments described here primary transcription was detected by dot blot hybridisation.

In the previous section it was shown that a neutralising monoclonal and a polyclonal antibody did not inhibit transcriptase activity significantly *in vitro*. The experiments below show the effect of the same neutralising monoclonal antibody on primary and secondary transcription *in vivo*.

2. RESULTS

(a) Analysis of RNA from BHK-21 cells inoculated with neutralised and non-neutralised virus

(FPV/R_M.PR/8_M) virus was incubated with either neutralising anti-HA IgG (HC2) or non-neutralising anti-NA (2R6) IgG and inoculated onto BHK-21 cells. These cells are permissive for influenza virus replication (Kelly *et al.*, 1974) and neutralisation titres are the same as those obtained using CEF cells (Methods and Optimisations Part 7). After 3 hours at 37°C RNA was extracted and analysed by dot-blot hybridisation using radiolabelled vRNA to detect cRNA. Figure 51 shows that viral cRNA was present in cells inoculated with anti-NA treated (infectious) virus whereas, in cells inoculated with neutralised virus, no viral RNA was detectable above background, i.e. that for non-infected cells. Thus, using a different technique, virus and cells from Possee *et al.* (1982) these results confirm that neutralisation results in a lack of secondary transcription.

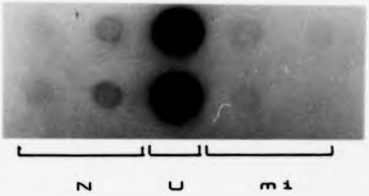


Figure 51: Analysis of RNA extracted from BHK-21 cells after inoculation and incubation for 3 hours at 37°C

(FPV/R.PR/8) virus was incubated with neutralising anti-HA IgG (HC2) or non-neutralising anti-NA IgG (2R6). The former neutralised infectivity by 99.99%. BHK-21 cells (10^{7} cells/dish) were inoculated with virus-antibody mixtures in PBS (equivalent to an moi of 100 for non-neutralised virus), or mock-inoculated with PBS alone, and incubated on ice for 1 hour. The cells were washed and incubated in tissue culture medium for 3 hours at 37°C. RNA was extracted, denatured with 50% formamide (65°C, 10 minutes) and analysed for virus-specific cRNA by dot-blot hybridisation (approximately 100 µg RNA/duplicate). The autoradiograph was exposed for 3 hours at -70°C with an intensifying screen.
Cells were inoculated with: N= neutralised virus;
U= non-neutralised virus and
mi= mock-inoculated cells.

(b) Analysis of RNA from cycloheximide-treated BHK-21 cells inoculated with non-neutralised and neutralised virus

To determine whether neutralised virus directed primary transcription, BHK-21 cells were inoculated as described above but in the presence of cycloheximide. Figure 52 is a dot blot hybridisation analysis of RNA extracted from cells after incubation at 37°C for the times shown. mRNA produced by primary transcription was detectable in cells inoculated with infectious virus after 1 hour and accumulated with time. Viral mRNA was not detectable in the RNA from cells inoculated with neutralised virus. Thus, neutralisation inhibits primary transcription, the first synthetic event of replication.

3. DISCUSSION

Primary transcription was analysed using cycloheximide to limit secondary transcription in BHK-21 cells inoculated with virus. The results show that neutralised virus is transcriptionally silent. The RNA of influenza virus neutralised with polyclonal or monoclonal IgG directed to HA enters the nuclear fraction of CEF and BHK-21 cells with the same kinetics as infectious virus (Possee and Dimmock, 1981; Possee *et al.*, 1982; Dimmock *et al.*, 1984; Figure 54(b), Results and Discussion Section IV). Taken together this information suggests that virion RNA-containing sub-viral particles from neutralised virus reach the nucleus but are unable to begin transcription, consistent with the hypothesis that neutralisation is a consequence of inhibition of transcriptase activity. However, the neutralising monoclonal antibody used does not lower transcriptase activity *in vitro*, showing that there is no strict correlation between inhibition of

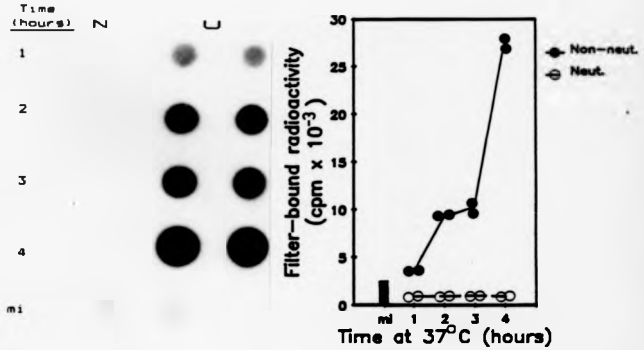


Figure 52: Analysis of RNA extracted from BHK-21 cells inoculated with antibody-treated virus in the presence of cycloheximide

Quantity of virus used and procedure as for Figure 51 except that cycloheximide was included in the PBS and tissue culture medium at 200 µg/ml (Avery and Dimmock, 1975). This inhibited protein synthesis by 98.9% (see Part 15, Methods and Optimisations). Cells were harvested after incubation at 37°C for the times shown. RNA was extracted and 18 µg of RNA per duplicate was applied to the membrane after denaturation with 50% formamide/6% formaldehyde for 10 minutes at 55°C. The autoradiograph was exposed for 3 hours with an intensifying screen at -70°C. The infectivity of neutralised virus (○) was 99.99% lower than the control (●). mi= mock-inoculated cells. Individual dots were cut from the membrane and the radioactivity determined.

transcriptase activity *in vitro* and *in vivo* as postulated by Possee (1981). Additionally, because the lipid envelope of neutralised virus was found in the cytoplasm whilst the genomes accumulated in the nucleus, it was assumed that uncoating of neutralised virus occurs normally. The relevance of uncoating to neutralisation is considered in the following section.

Figure 51 confirms and extends the findings of Possee *et al.* (1982) that, at the time when secondary transcription is occurring in a normal infection, neutralised virus produces no detectable cRNA. Since essentially the same results were obtained with BHK-21 cells and a reassortant virus as Possee *et al.* (1982) obtained with FPV and CEF cells, this suggests that the mechanism may apply generally to other influenza virus-cell combinations.

SECTION IV: The effect of neutralisation on uncoating of influenza virus *in vivo*

1. INTRODUCTION

The initial stages of infection by influenza virus are attachment, uptake, uncoating and transport of the virion RNA and associated proteins to the nucleus. These are followed by primary transcription, the first synthetic event. For virus neutralised with IgG, attachment, uptake and nuclear accumulation occur normally (Possee and Dimmock, 1981; Possee *et al.*, 1982; Dimmock *et al.*, 1984) and the lipid component of the envelope remains in the cytoplasm (Possee, 1981). However, no primary transcription is detectable (Results and Discussion, Section III). This could be the result of direct inhibition of transcriptase activity (Possee *et al.*, 1982) but could equally be due to incomplete uncoating with the result that the transcriptase enzyme is not activated.

The process of uncoating of influenza virus *in vivo* is poorly documented. Use has been made of amantadine, and an analogue rimantadine, which inhibit the uncoating process (Part 6, General Introduction). The evidence suggests that uncoating is a two stage process. The first occurs when the low pH of the endosomal vesicle induces a conformational change in the HA and as a consequence the viral and endosomal membranes fuse. This liberates sub-viral particles into the cytoplasm. In the presence of rimantadine these sub-viral particles, which possess M and NP proteins (M1-RNP), are found in the nuclear-associated cytoplasm and nucleus (Bukrinskaya *et al.*, 1982). In the absence of the

drug, RNP complexes are seen in which only NP is detectable. Thus, the secondary stage of uncoating involves loss of M protein from sub-viral particles yielding RNP complexes (Bukrinskaya et al., 1982). Infection of cells by influenza virus is accompanied by the virion RNA becoming sensitive to RNase digestion, but in the presence of rimantadine little increase in RNase sensitivity is seen (Koff and Knight, 1979). Taken together with the data of Bukrinskaya et al. (1982) this implies that the secondary stage of uncoating must occur for the virion RNA to become susceptible to RNase i.e. RNase sensitivity is an indirect measure of secondary uncoating.

In this section uncoating of influenza virus was investigated by measuring RNase sensitivity of the input genome using a method based on that of Koff and Knight (1979). BHK-21 cells were inoculated with [^{32}P]-labelled virus and, after the required incubation time, the cells were disrupted with ultrasound. RNases were added to the lysates and the quantity of intact virion RNA measured by TCA precipitation. The results suggest that neutralisation interferes with secondary uncoating.

2. RESULTS

(a) No increase in RNase sensitivity of vRNA occurs in cells held on ice after inoculation

To synchronise influenza virus infection inoculation is carried out at temperatures between 0 and 5°C and Figure 53 shows that the virion RNA did not become sensitive to digestion after inoculation for 1 hour and incubation for 3 hours on ice. It is possible that virus was attaching to, but not entering, cells and therefore uptake of virus under these conditions was investigated

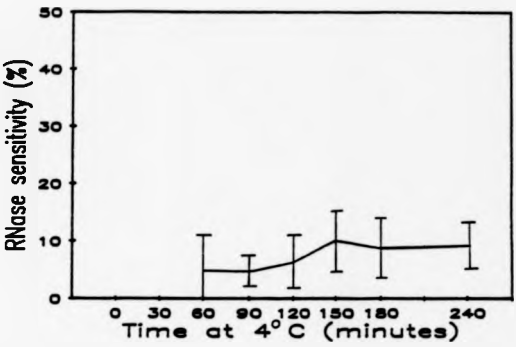


Figure 33: RNase sensitivity of virus inoculated onto BHK-21 cells and held on ice

[^{32}P]-FPV (100 µl) in PBS was inoculated onto monolayers of BHK-21 cells (6 x 10^6 cells/dish) and incubated for 60 minutes. Each 100 µl contained 8.9 x 10^7 pfu, 4.9 x 10^3 HAU and 7.5 x 10^5 TCA-precipitable cpm. Cells and solutions were held on ice throughout. Excess inoculum was removed and the monolayers washed twice with 1 ml of PBS, 1 ml of PBS was added and incubation continued. Each point is the mean for four replicate monolayers and a bar indicates the standard error of the mean ($p = 0.05$, $n-1$ degrees of freedom).

by cell fractionation. Figure 54(a) shows validation of the fractionation of BHK-21 cells by the nuclear monolayer technique (Dimmock et al., 1984) and demonstrates that approximately 85% of cellular RNA was in the cytoplasmic fraction. In a parallel experiment, Figure 54(b), approximately 70% of the virion RNA taken up by cells had migrated to the nuclear fraction after incubation for 90 minutes on ice and essentially the same result was obtained with non-neutralised and neutralised virus. This suggests that virus taken up by cells undergoes primary uncoating, resulting in the appearance of virion RNA in the nucleus, but that secondary uncoating does not occur during incubation on ice. Incubation at this temperature provides a negative control, useful since amantadine does not inhibit uncoating of FPV/Rostock (Beyer et al., 1986).

In order to check the maximum attachment to cells held on ice, virus was incubated with cells for up to 180 minutes (Figure 55). A plateau was reached in about 90 minutes and, as no change in RNase sensitivity occurred under these conditions (Figure 53) this time was used for subsequent inoculations.

(b) Kinetics of uncoating of influenza virus in BHK-21 cells

Figure 56 shows that the virion RNA of non-neutralised virus became progressively more RNase sensitive up to 90 minutes at 37°C with a maximum value of 41%. In three other experiments the extent of RNase sensitivity varied up to a maximum value of 58% (Figure 57).

Results are also shown for neutralised virus. At 60, 90 and 120 minutes post-infection the ratios of RNase sensitivity of

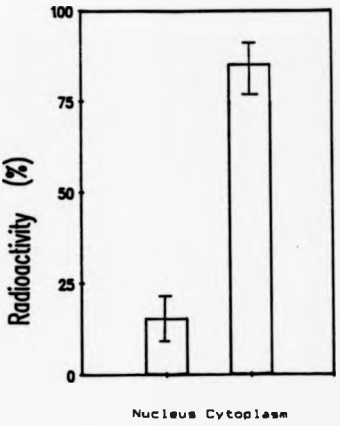
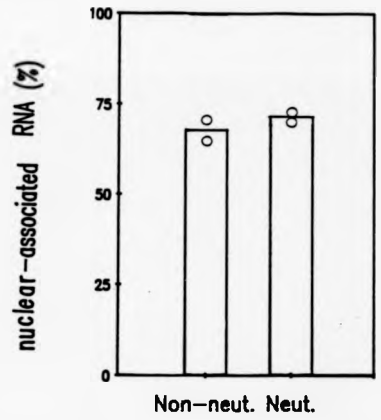


Figure 54: (a) Distribution of radioactively labelled RNA (cytoplasmic marker) in BHK-21 cells fractionated by the nuclear monolayer method

Sub-confluent BHK-21 cells (10^6 cells/dish) were incubated for 24 hours at 37°C in tissue culture medium containing [^{3}H]-uridine (1 μCi/ml), the medium was replaced with medium lacking the radioactive label and incubation continued for a further 24 hours. This regimen labels cellular mRNA and rRNA (Possee, 1981). The cells were fractionated by the nuclear monolayer method and the TCA-insoluble radioactivity determined. The value for each fraction is expressed as a percentage of the total: The mean for four monolayers is plotted and a bar indicates the standard error of the mean ($p = 0.05$, $n-1$ degrees of freedom).



(b) The proportion of [^{32}P]-virion RNA of non-neutralised and neutralised virus in the nuclear fraction of BHK-21 cells

[^{32}P]-FPV was incubated with either neutralising anti-HA (HC2) or non-specific (1B5/1) monoclonal IgG. The infectivity of the neutralised virus was 99.9% lower than the control. BHK-21 cells (10^6 cells/dish) were incubated for 48 hours at 37°C, chilled, inoculated with virus (100 µl) and held on ice for 90 minutes. Each 100 µl contained 7.5×10^6 pfu, 38 HAU and 6.9×10^6 cpm. After 90 minutes 52% was cell associated. The ordinate shows the proportion of cell-associated virion RNA in the nuclear fraction. The values plotted are the means for duplicate monolayers (○)

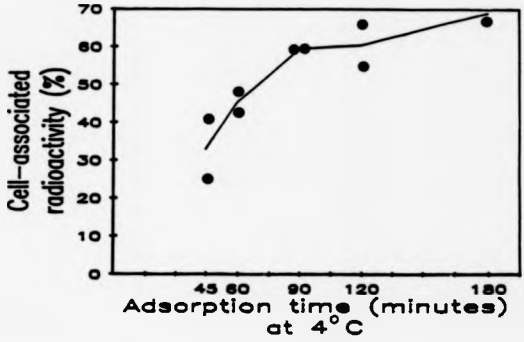


Figure 55: Attachment of FPV to BHK-21 cells held on ice

[^{32}P]-FPV was inoculated onto monolayers of BHK-21 cells (6 x 10^{6} cells/dish) held on ice. Each 100 µl contained 3.5 x 10^{7} pfu, 1.4 x 10^{3} HAU and 1.1 x 10^{5} cpm. After incubation for the times shown the monolayers were washed twice with 1 ml of cold PBS. The TCA-precipitable radioactivity associated with cells was determined and is expressed as a percentage of that inoculated.

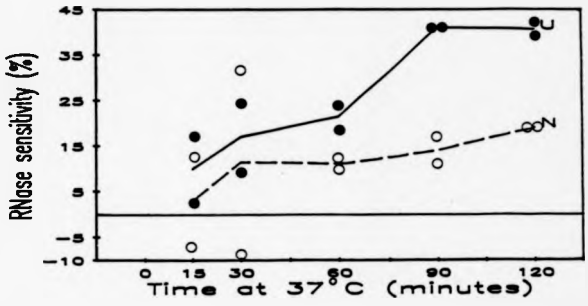


Figure 56: Acquisition of RNase sensitivity of non-neutralised and neutralised virus after inoculation onto BHK-21 cells

^{32}P]-FPV was incubated for 1 hour at 25°C with monoclonal neutralising IgG (N) (HC2) or non-specific IgG (U) (185/1). The former neutralised virus by 99.99%. Virus-antibody mixtures were inoculated onto duplicate BHK-21 cell monolayers (6 x 10^6 cells/dish). Each 100 µl initially contained 5.1 x 10^7 pfu, 2.8 x 10^3 HAU and 4.3 x 10^3 cpm. Inoculation and incubation were carried out at 37°C, thus zero time is the time at which virus was added to the cells. The values for RNase sensitivity of the virion RNA in lysates of individual monolayers are shown and the means plotted.

non-neutralised virus were (22/11) 2, (41/9) 4.6 and (40/19) 2.1 respectively. The RNase sensitivity of neutralised virus was lower at each time than that of non-neutralised virus suggesting that neutralisation was preventing uncoating. To determine if these differences were statistically significant further experiments were performed and the effect of different quantities of neutralising antibody on uncoating were assayed in quadruplicate.

(c) RNase sensitivity of neutralised virus

Figure 57 shows that the RNase sensitivity of non-neutralised virus was (58/8) 7.3-fold higher after incubation at 37°C. In contrast, treatment of virus with neutralising antibody reduced RNase sensitivity. At the three highest concentrations of antibody the RNase sensitivity of virus incubated at 37°C did not differ significantly from virus inoculated onto cells held on ice. The neutralisation titres for all these samples were above 99.9%, as shown. The RNA of virus neutralised by 79%, however, was more susceptible to RNase, but still less susceptible than that of non-neutralised virus. Thus there is a correlation between neutralisation and uncoating, measured as RNase sensitivity.

This relationship is supported by another experiment, in which the results for RNase sensitivity and neutralisation are plotted against dilution of antibody, Figure 58. RNase sensitivity increased as the quantity of neutralising antibody was reduced though neutralisation dilutes out a half-log dilution prior to the point at which RNase sensitivity becomes indistinguishable from the control.

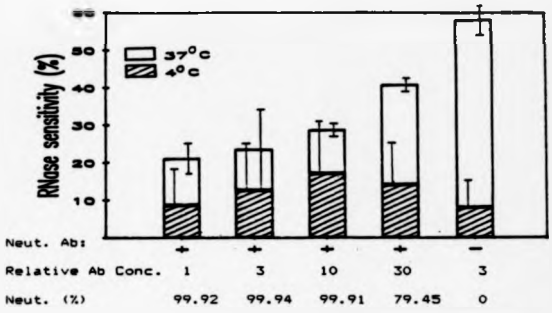


Figure 57: The effect of different dilutions of neutralising antibody on uncoating of influenza virus

[^{32}P]-FPV was incubated for 1 hour at 25°C with dilutions of neutralising monoclonal IgG (HC2) which lowered infectivity as indicated. The non-neutralised control contained non-specific IgG at the relative concentration shown. Virus-antibody mixtures were cooled and 100 µl inoculated onto 6 replicate monolayers of BHK-21 cells (6 x 10^6 cells/dish). Each 100 µl initially contained 6.8 x 10^5 pfu, 2.7 x 10^2 HAU and 2.1 x 10^4 cpm. RNase sensitivity was assayed for 2 dishes after 90 minutes on ice (hatched sections of bars). The remaining four dishes were transferred to 37°C for a further 90 minutes before RNase sensitivity was assayed. For each sample the fraction of radioactivity resistant to boiling in TCA was determined and subtracted (Part 1B, Methods and Optimisations). The ordinate shows the mean corrected values and a bar indicates the standard error of the mean (p= 0.05, n-1 degrees of freedom).

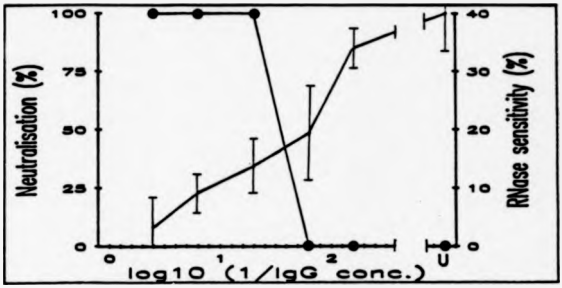


Figure 58: The relationship between RNase sensitivity of the genome of virus inoculated onto BHK-21 cells, neutralisation titre and dilution of neutralising antibody

Protocol as for Figure 57 except that each 100 µl of virus-antibody mixture initially contained 8.9×10^{5} pfu, 2.4×10^{3} HAU and 1.7×10^{6} cpm. The quantity of non-specific IgG used for the control was equivalent to 1.3 $\log_{10}$ (1/IgG dilution). The values shown are the mean corrected RNase sensitivities for quadruplicate monolayers after inoculation of virus on ice for 90 minutes followed by incubation at 37°C for a further 90 minutes.

3. DISCUSSION

Koff and Knight (1979) assayed the sensitivity of WSN virion RNA during infection of MDCK cells and showed that after transfer to 37°C the virion RNA remained RNase resistant for a period of 20 minutes and then became progressively susceptible to digestion. The maximum sensitivity was seen after 90 minutes when 50% of the virion RNA was digested and incubation for a further 30 minutes did not appreciably increase the sensitive fraction.

The results presented here for FPV inoculated onto BHK-21 cells show similar kinetics for the development of RNase sensitivity (Figure 56). It was found that only approximately half of the virion RNA became RNase sensitive, also in agreement with Koff and Knight (1979). That only half the virion RNA becomes susceptible may be a consequence of determining RNase sensitivity by TCA precipitation. Nucleic acid molecules as small as 20 nucleotides are precipitated by this method (Maniatis et al., 1982) and the existence of virion RNA in RNP complexes could confer protection. Additionally this might explain why only approximately half of the input virion RNA becomes sensitive to RNase during a normal infection. Assay of RNase sensitivity could be refined by Northern Blot analysis of virion RNA using a probe specific for a single genome segment. Cell lysates could be treated with RNase and the RNA extracted and electrophoresed. After blotting and hybridisation the differences in the strength of the signal obtained at the position of the undigested gene would give a measure of RNase sensitivity. Using an assay such as this, the proportion of virion RNA that became sensitive to RNase digestion during a normal infection may be expected to approach

100%.

Susceptibility of virion RNA to digestion by RNase after infection was used to measure uncoating. The results show that neutralisation limits the extent to which virion RNA becomes RNase sensitive. This suggests, by analogy to the studies employing rimantadine (Koff and Knight, 1979; Bukrinskaya et al., 1982), that the stage of infection compromised by neutralisation is at, or prior to, secondary uncoating. Studies demonstrate that virus neutralised with IgG undergoes primary uncoating with virion lipid remaining in the cytoplasm (Possee et al., 1982) whilst virion RNA is transported to the nucleus (Possee and Dimmock,1981; Possee et al.,1982; Dimmock et al., 1984; Figure 54(b)). However, increase in RNase sensitivity was limited (Figures 57 and 58) and no primary transcription was detectable (Results and Discussion Section III). Therefore, it is postulated that neutralisation prevents secondary uncoating which in turn results in failure of the virus to direct transcription.

A relationship between neutralisation and uncoating is implied by the results showing that RNase sensitivity increased as the quantity of neutralising antibody used to treat virus was reduced. However, strict proportionality was not seen as there was still reduced RNase sensitivity at the dilution of neutralising antibody at which neutralisation ceased. This may also be a consequence of measuring RNase sensitivity by TCA precipitation, as discussed above.

Virion RNA did not become sensitive to RNase when inoculated onto cells held on ice. At temperatures of 0-5°C influenza viruses attach to cells (reviewed by Dimmock, 1982; Matlin et al. 1981;

Yoshimura et al., 1982; Dimmock et al., 1984; Richman et al., 1986) and undergo uncoating (Kato and Eggers, 1969) with virion lipid (Hudson et al., 1978; Possee et al., 1982) and glycoproteins (Hudson et al., 1978; Possee, 1981) remaining in the cytoplasm. Virion RNA (Stephenson and Dimmock, 1975; Mark et al.,1979; Possee and Dimmock, 1981; Possee et al.,1982; Dimmock et al.,1984; Figure 54(b)) and core proteins (Hudson et al., 1978) enter the nucleus but there is no primary transcription. These results can be reconciled with the failure of virion RNA to become sensitive to RNase if it is assumed that primary but not secondary uncoating occurs at low temperature. It may be that attachment, penetration and primary uncoating occur by energy-independent processes, but energy is required for secondary uncoating. The interaction of influenza virus with cells at low temperature is still a subject of contention. Bukrinskaya et al. (1982) state that both stages of uncoating occur at 4°C, but present no data. Some authors dispute that influenza virus enters cells at low temperature (Matlin et al., 1981; Yoshimura et al., 1982; Richman et al., 1986) but the reason for these differences is unclear. With WSN and MDCK cells attachment but not penetration was observed by Matlin et al. (1981) and Yoshimura et al. (1982) but attachment and penetration has been described for the same virus-cell combination by Mark et al. (1979) and Bukrinskaya et al. (1982). The amount of virus inoculated onto cells also appears to be of little consequence in this regard: Matlin et al. (1981) used a ratio of 4 particles per cell and found that only 4% of the cell-associated virus was resistant to removal by neuraminidase and therefore assumed to have been internalised. Richman et al. (1986) inoculated LLC-MK2 cells with 2.4 particles per cell and could detect no uptake into intracellular compartments. Other workers have used larger

quantities of virus: Mark et al. (1979) detected 10^3 molecules of WSN virion RNA per nucleus in MDCK cells inoculated at an moi of 30-60 (probably 3 x 10^3 to 6 x 10^3 particles per cell) whereas, with the same virus-cell combination and ratios of 10^4 and 10^5 particles per cell, Yoshimura et al. (1982) reported no penetration. Finally, penetration does not seem to be dependent on whether a temperature of 0 or 4°C is used. Attachment only was observed by Matlin et al. (1981) at 0°C and by Yoshimura et al. (1982) at 4°C, whereas accumulation of virion RNA in nuclei has been shown to occur in cells held on ice (Figure 54(b)) and at 4°C (Mark et al., 1979).

SECTION V: Changes in virion structure induced by neutralising antibody

1. INTRODUCTION

The fact that antibody interrupts infection after entry of influenza virus into cells implies that neutralisation is the consequence of some change in the internal structure of the virion induced by antibody binding to the HA. The experiments described below were prompted by observation of differences in the sedimentation properties of virion components after neutralised and non-neutralised virus had been treated with detergent (A.S.Carver and N.J.Dimmock, unpublished results). Detergent treatment of non-neutralised virus produced two peaks when analysed by sucrose density centrifugation. One peak consisted predominantly of the glycoproteins, HA and NA and the other, the core band, contained M, NP and the polymerase proteins. However, after detergent treatment of virus neutralised with a monoclonal anti-HA IgG (HC2), the glycoprotein band lacked HA, and this was not detectable in the other gradient fractions. It was assumed that the missing protein had pelleted because when radiolabelled virus was used, more radioactivity was associated with the tube bottoms from gradients loaded with detergent-treated, neutralised virus than non-neutralised virus. Whilst it was possible that the HA had been aggregated by antibody this was not the only explanation since when virus is reacted with sufficient antibody (0.4 HIU monoclonal antibody/HAU of virus) monodispersed virus particles coated in antibody are seen (Figures 59 and 60). Cross-linking did occur but at antibody:virus ratios 10 to 1,000-fold lower (Taylor *et al.*,

Figure 39: Electron micrographs of influenza virus and influenza virus saturated with neutralising antibody

(a) Influenza virus

(b) Influenza virus saturated with neutralising antibody (HC2)

Electron micrographs kindly provided by Dr.S. Armstrong

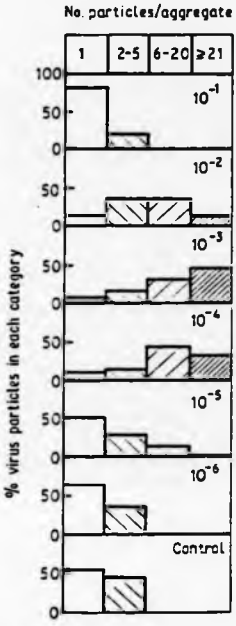


Figure 60: The effect of concentration of neutralising antibody on aggregation of influenza virus particles

Results obtained in an independent study and reported by Taylor et al. (1987) showing that virus particles are not aggregated when reacted with high concentrations of neutralising monoclonal antibody. Various dilutions of purified anti-HA IgG (HC2) were incubated with 2×10^8 HAU of virus for 1 hour at 25°C and samples were taken for electron microscopy. 10^{-1} dilution of IgG = 6 µg IgG (1.7×10^{11} HIU)/4 x 10^8 HAU of virus in a volume of 20 µl.

1987; Figure 60). Consequently cross-linking is eliminated if sufficient antibody is used. For any monoclonal anti-HA antibody there are three binding sites per HA trimer but virus in solution binds only one antibody molecule per spike (Taylor et al., 1987). Since detergent treatment of virus might expose the other two sites, in the following experiments a 10-fold excess of antibody (at least 10 HIU IgG/HAU) was used to ensure saturating conditions. Two different neutralising monoclonal IgGs were used, HC2 and HC61. Both map to different epitopes within the antigenic site corresponding to site A of the H3 subtype (J.W.McCauley, H.P.Taylor and N.J.Dimmock, unpublished results; Figure 2, General Introduction). The non-neutralising antibody was a monoclonal anti-HA IgG (185/1) that does not bind to the strain of virus used.

2. RESULTS

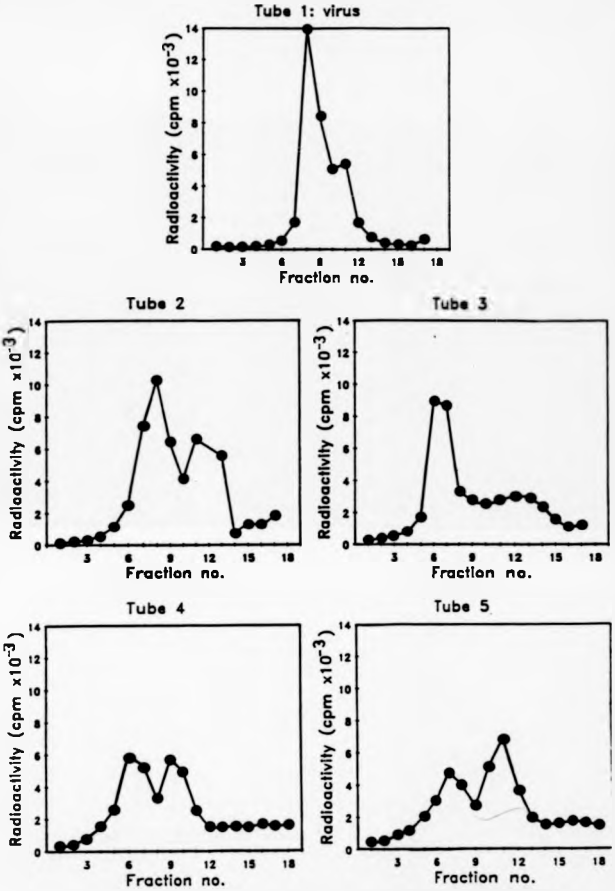
(a) Optimisation of detergent concentration

Previous work indicated the importance of the quantity of detergent used to disrupt virus (A.S.Carver and N.J.Dimmock, unpublished results). They used either egg-grown, unlabelled virus or [^{35}S]-methionine labelled virus to which unlabelled virus was added so that the minimum concentration was 4×10^3 HAU of virus/300 µl; 1 µl of detergent (10% NP40 and 0.25% Triton X-100) was used per 10^3 HAU of virus. Detergent treatment was carried out in 300 µl of PBS on ice for 4 minutes. The incubation time and temperature are critical. Incubation with the same quantity of detergent for longer periods and at higher temperatures results in disintegration of viral cores. However, once banded, and thereby separated from detergent, cores were

Figure 61: Distribution of radioactivity in gradients loaded with virus and detergent-treated virus

Virus in 300 µl of PBS was incubated with the quantities of detergent shown for 4 min on ice and layered on top of sucrose gradients (10%:40%:60% w/v) and centrifuged at 112,000g, 4°C, overnight. Gradients were fractionated and the radioactivity in each fraction determined. Fraction 1 is the bottom of the gradient.
Key:

Tube no.	Conditions
1	virus no detergent
2	4×10^{-3} HAU virus + 4 µl detergent
3	10^{-3} HAU virus + 1 µl detergent
4	10^{-3} HAU virus + 3 µl detergent
5	10^{-3} HAU virus + 10 µl detergent


Tube 1: virus
Radioactivity (cpm x10-3)
Fraction no.
Tube 2
Radioactivity (cpm x10-3)
Fraction no.
Tube 3
Radioactivity (cpm x10-3)
Fraction no.
Tube 4
Radioactivity (cpm x10-3)
Fraction no.
Tube 5
Radioactivity (cpm x10-3)
Fraction no.

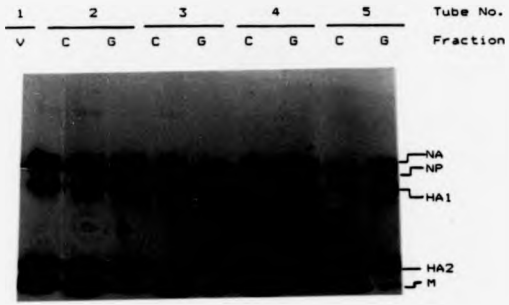
stable and rebanded homogeneously after storage at 4°C for a week (A.S.Carver and N.J.Dimmock, unpublished results). In the experiments described below it was possible to reduce the quantities of virus, allowing higher antibody:virus ratios to be achieved, by using radioactively labelled virus because the detection of radiolabelled proteins is more sensitive than staining of unlabelled proteins in polyacrylamide gels.

The optimum detergent concentration to disrupt 10^8 HAU of [^{35}S]-methionine virus was determined by analysing the products by sucrose density gradient centrifugation. With virus alone a single peak was obtained, whereas detergent treatment of virus with a small shoulder yielded two peaks (Figure 61). Fractions corresponding to the core and glycoprotein peak for each detergent concentration were analysed by PAGE (Figure 62). One incubation, tube 2, contained the quantity of virus and detergent used in previous work and tubes 3-5 were loaded with 10^8 HAU of virus after treatment with the quantities of detergent shown. The cores from tube 2 (2C) consist predominantly of NP and M with a much reduced amount of HA1 and HA2 compared to virus alone. Of the titrated samples the cores from tube 5 (5C) most closely resemble this pattern and therefore 3 µl of detergent/10^8 HAU was taken as optimum.

(b) Effect of detergent on antibody-treated virus

(i) Distribution of radioactivity in gradients

Figure 63 shows the distribution of radioactivity throughout gradients on which virus reacted with antibody and virus reacted with antibody and then detergent treated had been centrifuged.



C = core (higher density)

G = glycoprotein (lower density)

Figure 62: PAGE analysis of core and glycoprotein peaks from the detergent optimisation experiment

Equal quantities (approximately 10^{8} cpm) of core (higher density) and glycoprotein peaks were applied to the gel tracks as indicated. Tube numbers are as in Figure 61. A 10-30% gradient polyacrylamide gel was used under reducing conditions.

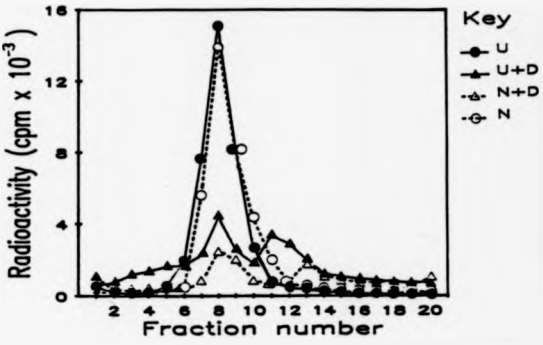


Figure 63: Distribution of radioactivity in gradients loaded with virus treated with antibody or antibody and detergent

[^{35}S]-FPV/R was incubated with neutralising or non-neutralising IgG (42 ug) at 25°C for 1 hour, chilled on ice and detergent (3 µl) or PBS added as shown. Detergent incubation and centrifugation were as described in Figure 61.

Non-neutralising IgG=185/1

Neutralising (anti-HA) IgG=HC61

Results from Experiment 2 (see Table 15)

Virus reacted with neutralising or non-neutralising antibody migrates as a single peak and reaches equilibrium at the same position. Detergent treatment of non-neutralised virus produces two peaks around fraction 8 (cores) and fraction 12 (glycoproteins). When virus was neutralised with monoclonal anti-HA IgG (HC61) prior to detergent treatment, two peaks were again discernible. Figure 64 shows the radioactivity associated with the bottoms of the centrifuge tubes. Comparison of the amount of radioactivity in the pellets from detergent-treated, non-neutralised virus with that from virus alone showed that detergent treatment increased the quantity of viral proteins in the pellet (5-fold). When neutralised virus was detergent-treated, 3.5-fold more radioactivity was associated with the pellets than was seen for non-neutralised virus after similar treatment. Table 15 shows that the results from two other experiments were similar. Figure 65 shows the distribution of viral proteins throughout gradients loaded with non-neutralised and neutralised virus after detergent treatment. The same quantity of radioactivity was applied to each track of the gels and 5 regions can be delineated: pellets; bottom fractions (fractions 1 to 6); cores (7 to 9); glycoproteins (11 to 14) and the top of the gradient (16 and above). Only in the core and glycoprotein fractions and pellets were any differences between neutralised and non-neutralised virus detectable. The protein composition of these fractions is considered below.

(ii) Composition of the glycoprotein peak and pellets

When virus is reacted with non-neutralising antibody and treated with detergent, the glycoprotein band contains HA1, HA2, NA/NP and M (Figure 66). The gels in Figure 67, where NP and NA are

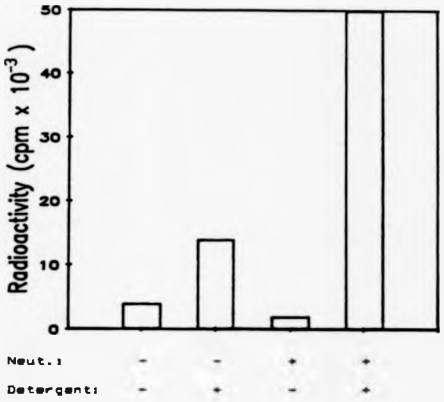


Figure 64: Radioactivity in the pellets after gradient centrifugation of virus reacted with monoclonal antibody with and without subsequent treatment with detergent

This figure represents the radioactivity associated with the pellets after harvesting the gradients shown in Figure 63.

Table 15: The amount of radioactivity in the pellets from gradients loaded with detergent-treated non-neutralised and neutralised virus

Expt No.	Neut. anti-HA MoAb	Radioactivity (cpm x 10^{-3})= Neut. virus pellet[a]	Unneut. virus pellet	Ratio N/U
1	HC2	88.0	27.0	3.3
2	HC61	50.3	14.5	3.5
3	HC61	13.5	5.0	2.7

Notes:

N = neutralised virus, U = non-neutralised virus.

a. Radioactivity for Experiments 1 and 2 was determined directly after immersing the tube bottoms in scintillation fluid. The results for Experiment 3 were determined after resuspending proteins from the tube bottoms.

b. The non-neutralising antibody was a non-specific monoclonal IgG (185/1)

Figure 65: PAGE analysis of fractions from sucrose gradients loaded with detergent-treated neutralised or non-neutralised virus

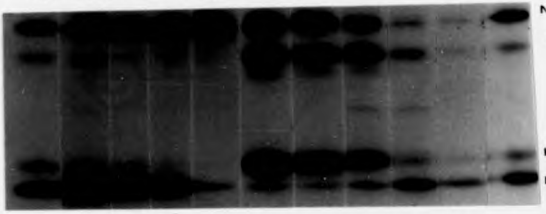
Key:v=virus
G=glycoprotein peak on sucrose gradient
C=core peak on sucrose gradient
P=pellet

Each track was loaded with 4 x 10^3 cpm.

Results from Experiment 3 (Table 15).

PAGE conditions as Figure 62.

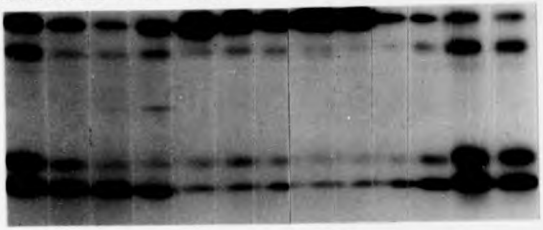
Non-neutralised virus (185/1)



V P 1 2 3 4 5 6 7 8 11 12 13 14 15 16 18 19 20 21 V

C G

Neutralised virus (HC61)



V P 1 2 3 4 5 6 7 8 9 11 12 13 14 15 16 17 18 19 20 21 V

C G

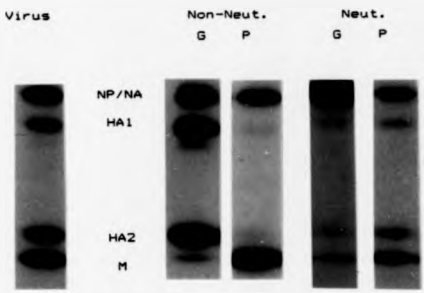


Figure 66: PAGE analysis of glycoprotein fractions and pellets from detergent-treated, non-neutralised and neutralised virus

Results from experiment 3 (Table 15 and Figure 65)

PAGE conditions as Figure 62

Key: G=glycoprotein; P=pellet

resolved, suggest that NA is the major constituent of the NP/NA band. Thus the quantities of NP and M in the glycoprotein band are much reduced compared to virus. When virus was reacted with neutralising antibody and then split with detergent a different pattern was obtained. Under these conditions the glycoprotein peak lacked HA1 and HA2 and this deficiency was specific and selective since NA, NP and M were still detectable. Figure 66 also shows an analysis of the protein composition of the pellets. The absence of HA1 and HA2 from the glycoprotein peak was accompanied by an increase in the proportion of these proteins in the pellet. Figure 68 demonstrates that neutralising IgG was also present in the pellet from a similar sample whereas non-neutralising antibody was absent from the corresponding pellet. Figure 67 shows the protein composition of glycoprotein bands from two other experiments and the results are the same as shown in Figure 66.

(iii) Composition of the core peak

No major differences in the protein constituents of cores derived from non-neutralised and neutralised virus were observed but one difference was noted. Comparison of the protein profiles of cores from neutralised and non-neutralised virus suggests that slightly more HA is retained on the cores from neutralised virus (Figures 69 and 70). This difference was consistent in 3 experiments with two neutralising monoclonal IgGs to different epitopes. However, only a small increase was seen and has been judged only by eye from autoradiographs; the levels of radioactivity in the gels were too low to allow quantitation by scintillation counting of excised bands.

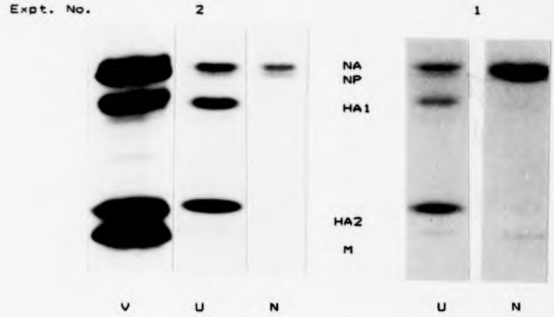


Figure 67: PAGE analysis of glycoprotein fractions from detergent-treated, non-neutralised and neutralised virus

Key: v=virus

glycoprotein fractions derived from U=non-neutralised virus
N=neutralised virus

PAGE conditions as Figure 62

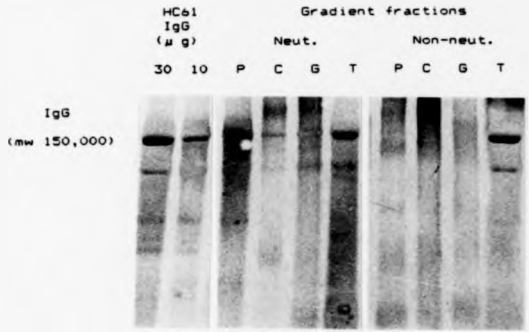


Figure 68: PAGE analysis of fractions of gradients loaded with detergent-treated non-neutralised and neutralised virus to detect IgG

The neutralising IgG used was HC61 and the non-neutralising IgG was 165/1. Gradient fractions from the top (T), glycoprotein (G) and core (C) bands were pooled and analysed with pellet (P) samples and IgG markers by electrophoresis under non-reducing conditions. The unlabelled proteins were detected by silver-staining. Influenza virus proteins do not migrate in the section of the gel shown (the viral proteins with molecular weights closest to 160,000 are the NA at 220,000 and the low-abundance polymerase proteins at approximately 90,000. None of these were present in detectable quantities.)

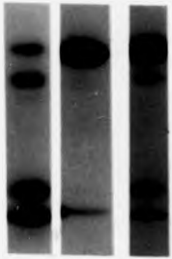


Figure 69: PAGE analysis of core fractions from detergent-treated, non-neutralised and neutralised virus

Results from Experiment 3 (Table 15 and Figure 65)

PAGE conditions as Figure 62

Non-neutralised virus (U) was reacted with non-specific IgG (185/1) or virus was neutralised (N) with anti-HA IgG (HC61)

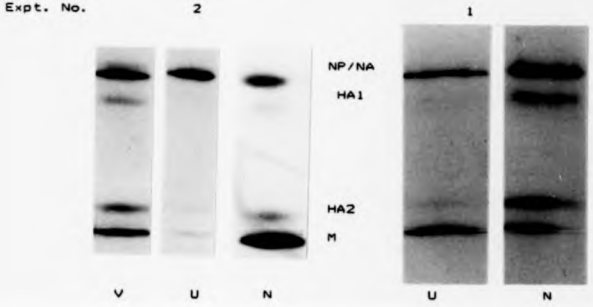


Figure 70: PAGE analysis of the core fractions from detergent-treated, non-neutralised and neutralised virus

Experiment numbers as Table 15

Key: v=virus
core fractions derived from U=Non-neutralised virus (185/1)
N=neutralised virus (HC2
IgG for Experiment 1and HC61 for Experiment 2)
PAGE conditions as Figure 62

The proportion of M protein in the core fractions was variable. Figure 70 shows that the quantity of M and NP/NA were approximately the same in the cores from non-neutralised virus produced in Experiment 1. In the corresponding sample from Experiment 2, however, NP/NA was in excess of M. The cores produced from neutralised virus in Experiment 2 have more M than NP/NA associated, but no enrichment in M content was observed in the cores from neutralised virus produced in Experiments 1 and 3.

3. DISCUSSION

Analysis of the proteins of detergent-treated, neutralised virus showed that HA was absent from the glycoprotein peak and that the pellets and core fractions were enriched for HA. The absence of HA from the glycoprotein peak accounts, at least in part, for reduction in radioactivity in this peak. Neutralising anti-HA specifically causes the HA to pellet.

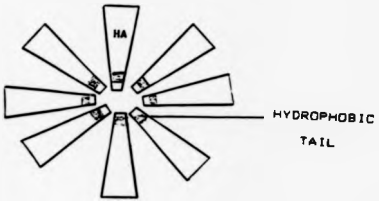
The reduced radioactivity in the core peak from detergent-treated, neutralised virus results from the loss of all components since the ratios of the remaining proteins are similar, although if anything, this fraction is enriched for HA. This apparent enrichment may be because more detergent is required to remove the HA from neutralised virus. Neutralisation may increase the stability of association of HA with internal virion components e.g. M1 or M2, or the decreased susceptibility of neutralised virus to detergent may be a consequence of saturating virus with antibody. These possibilities would also account for the increase in M protein associated with cores in one experiment. The increase in radioactivity associated with the

pellet is partly due to an increase in the proportion of HA, but the proportion of HA is clearly not increased 3-fold. Therefore the pellet must include cores or core components in addition to HA.

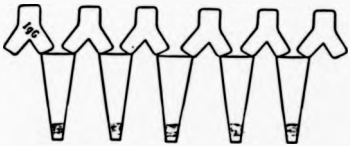
Detergent treatment of virus separates the glycoproteins from virus and when the detergent is removed glycoproteins associate together forming rosettes with hydrophobic termini at the centre (Wrigley, 1979). A mixture of HA and NA gives rise to mixed rosettes (Laver and Valentine, 1969) and a schematic diagram of an HA rosette is shown in Figure 71(a). They reach equilibrium in the sucrose gradients at a density of 1.16-1.18 g/ml whereas antibody, a glycoprotein with presumably the same density as HA and NA, remained at the top of the gradient (density < 1.05 g/ml) perhaps because it had not had time to reach its isopycnic point. The glycoproteins from detergent-treated, non-neutralised virus may associate together. The glycoprotein peak had haemagglutination activity (Figure 72) but monomeric HA, for example bromelain-released HA (Skehel et al., 1982), cannot agglutinate red blood cells. Even after detergent treatment, lipid may remain associated with the glycoproteins. Therefore, glycoproteins may associate because of the presence of residual lipid and association of their hydrophobic domains.

Under saturating conditions of antibody, only one IgG molecule per HA spike binds to influenza virus in solution (Taylor et al., 1987) and Figure 73 suggests ways in which this stoichiometry could be achieved using information from Wrigley et al. (1983). HA from neutralised virus pelleted to the bottom of the tube even though the maximum density of the gradient was 1.26 g/ml whereas the NA banded at 1.16-1.18 g/ml. Therefore, HA is rendered

Figure 71: Model to account for pelleting of the HA after interaction with anti-HA IgG



(a) Free HA in aqueous solution form rosettes with the hydrophobic tails (C-termini of HA2) buried in the centre.



(b) As a result of a conformational change or steric hindrance imposed by neutralising IgG, the hydrophobic tails of HA are exposed to the solvent.

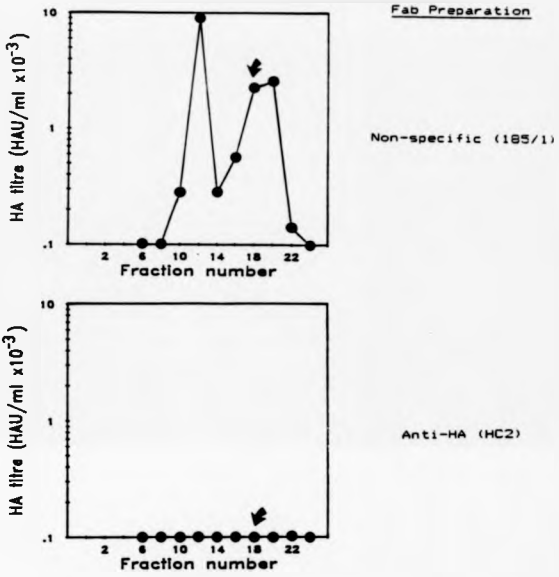


Figure 72: Haemagglutination assay of fractions from sucrose gradients loaded with virus incubated with Fab fragments and subsequently detergent treated

Fractions 10-12 = core peak
15-18 = glycoprotein peak

10^8 HAU of FPV/R was incubated for 1 hour at 25°C with 35 ug of anti-HA (HC2) or non-specific (185/1) Fab fragments as shown. Virus-Fab mixtures were detergent treated and centrifuged as described in Figure 61. The HA titre of the fractions were determined. Arrows indicate the fractions analysed by PAGE (Figure 74) which contain HA1 and HA2 polypeptides.

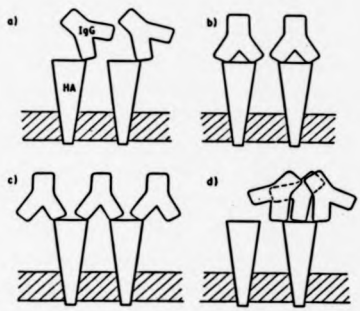


Figure 73: Interaction of IgG molecules with influenza virus HA spikes (drawn approximately to scale) showing some of the ways in which IgG may react with the three identical antigenic sites on each HA trimer under the saturating conditions of one IgG molecule/ spike. (a) to (c) represent random distributions of antibody over the surface of the virus. In (a) two antigenic sites per HA spike are unoccupied by IgG and in (b) and (c) one site per spike is unoccupied. (d) represents a hypothetical random distribution. Taken from Taylor et al. (1987).

insoluble by combination with neutralising antibody. This is important if the change (e.g. in conformation or charge) is specifically induced by neutralising antibodies, but may simply be an example of immune-precipitation (reviewed by Steensgaard, 1984; see Part 4 below).

Preliminary evidence using antibody fragments suggests that bivalency is required to cause the HA to pellet. IgG was fragmented using pepsin followed by cysteine reduction (Methods and Optimisations, Part 6). This yielded a mixture of $F(ab')_2$ and Fab' fragments, called Fab preparation. For the experiment shown using fragments of HC61 the ratio of Fab' to $F(ab')_2$ was 4:1 (Appendix A). When virus was reacted with Fab preparations derived from HC2 or HC61, little extra radioactivity was precipitated (Table 16) and HA was still present in glycoprotein fractions (Figure 74), even though saturating amounts of these Fab preparations were used. No haemagglutination activity was detectable in the fractions of the gradient loaded with virus reacted with specific anti-HA Fab fragments demonstrating that these fragments bound to the virus (Figure 72). Obviously, with monovalent Fab fragments inter-spike cross-linking and intra-spike cross-linking cannot occur suggesting that bivalency may be required to cause precipitation of HA. The interpretation of these results is equivocal, however, because Fab fragments do not neutralise influenza virus (Kida et al., 1985; Yoden et al., 1985; R.J.Rigg and N.J.Dimmock, Appendix A) and may for this reason cause no conformational change.

Future work should be directed to determining if pelleting of the HA correlates with neutralisation.

Table 16: The quantities of radioactivity in the pellets after centrifugation of virus reacted with Fab fragments and treated with detergent

Experiment	Radioactivity (cpm x 10^{-3}),[a] Anti-HA Fab[b] (clone)	Non-specific Fab[b] (185/1)	Ratio Specific/non-specific
A	19.5 (HC61)	14.5	1.3
B	28.0 (HC2)	23.5	1.2

Notes:

a. Radioactivity in the pellets was determined after immersing the tube bottoms in scintillation fluid.

b. Fab fragments were derived from the monoclonal IgGs indicated. Neutralisation titres were determined after adding rabbit anti-mouse IgG (RAM) and under these conditions the anti-HA Fabs reduced infectivity. However, Fab preparations alone neutralised infectivity relatively poorly (see General Discussion and Appendix A) and therefore the infectivity of virus reacted with Fab fragments here was probably little changed compared to virus reacted with non-specific Fab fragments. Fab preparations inhibit HA activity (Figure 72) indicating that Fab fragments bind to virus.

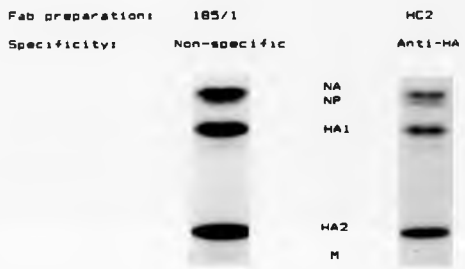


Figure 74: Comparison of glycoprotein bands derived from virus reacted with non-specific and specific Fab preparations

Results from Experiment B (Table 16)

The distribution of HA activity throughout these gradients is shown in Figure 72

PAGE conditions as Figure 62

4. MODELS TO ACCOUNT FOR PRECIPITATION OF THE HA

(a) Precipitation of HA may be the result of a conformational change specifically induced by neutralising antibody

Antibody can induce conformational changes in antigens (see General Introduction, Part 16(b)) and this may result in exposure of hydrophobic regions so that the antigen-antibody complex is insoluble (Steensgaard, 1984). For the precipitation of HA-IgG complexes to be relevant to neutralisation, whatever the change is that causes precipitation must be induced by neutralising, but not non-neutralising, antibodies. Therefore, to determine if precipitation of HA is neutralisation-specific, solubility of IgG-HA complexes produced using a panel of neutralising and non-neutralising anti-HA antibodies should be studied.

It is postulated that neutralising antibody induces a conformational change in the HA of influenza virus causing transmission of a transmembrane signal which modifies some internal virion component rendering the virus non-infectious (Possee et al., 1982; Taylor, 1986; this thesis). Using isolated HA, Kida et al. (1985) and Wharton et al. (1986) examined the ability of HA reacted with anti-HA antibody to undergo the conformational change induced by mildly acidic pH. Both groups of workers found that HA could not undergo this conformational change and their results provide no evidence for a conformational change in HA induced by reaction with antibody. Thus, these in vitro studies do not support the hypothesis that neutralising antibody induces conformational changes in the HA. At pH 5 the HA undergoes a major conformational rearrangement and it may be that the methods which detect this change are too insensitive to

detect the putative change induced by antibody.

(b) Precipitation of HA is antibody, but not neutralisation, dependent

At least two neutralisation-irrelevant mechanisms could account for precipitation of the HA.

(i) Immune-precipitation

This is a general term for reactions resulting in the formation of insoluble antibody-antigen complexes. Even the prototype of immune-precipitation, the precipitin reaction of rabbit IgG, is poorly understood, but insoluble complexes are thought to arise through exposure of hydrophobic regions in antigen or antibody, or by masking of hydrophilic regions on the antigen (Steensgaard, 1984).

The Fc region of antibody also plays an important part in the process of immune precipitation. Therefore, the role of immune precipitation in the system described here could be assessed by detergent treating virus neutralised with $F(ab')_2$ fragments (Moller, 1979), or antibody with altered Fc reactivity as a result of acylation (Nisonoff and Pressman, 1958).

(ii) Anti-HA may prevent rosette formation

If antibody bound to the HA as shown in Figure 73(c), the antibody might impose steric constraints and prevent the hydrophobic tails (which may or may not be associated with lipid) from associating after detergent treatment (Figure 71(b)). The

low solubility of the hydrophobic domains would render the antibody-HA complexes insoluble. The size of IgG-HA complexes released from neutralised virus by detergent treatment could be determined by velocity gradient centrifugation (Moller and Steensgaard, 1979). If the complexes were found to be IgG-HA monomers (Figure 73 (a),(b),(d)) the model shown in Figure 71(b) could be discounted.

GENERAL DISCUSSION

Possee *et al*. (1982) proposed that neutralisation of influenza virus by anti-HA IgG occurred after:

(1) induction of a conformational change in the HA,
(2) transmission of the change across the viral envelope which
(3) resulted in the inhibition of the virion transcriptase activity and hence, loss of infectivity.

The results presented in this thesis are consistent with this hypothesis and account for the inhibition of transcriptase activity. The results demonstrate that *in vivo*, neutralised influenza virus fails to direct primary transcription (Results and Discussion, Section III) (whereas Possee *et al*. (1982) had examined secondary transcription). Further, uncoating of neutralised virus is incomplete (Results and Discussion, Section IV)(this was not known by Possee *et al*. (1982)) and it is postulated that this results in failure of the transcriptase enzyme of neutralised virus to become active in cells. Possee *et al*. (1982) also reported inhibition of transcriptase activity *in vitro* by antibody, but not all neutralising IgGs inhibit transcriptase activity *in vitro* (Shimizu *et al*., 1985; Results and Discussion, Section II).

Figure 75 is a diagram showing our current theory for the mechanism of neutralisation of influenza virus by IgG (compare with Figure 15, General Discussion). Virus saturated with

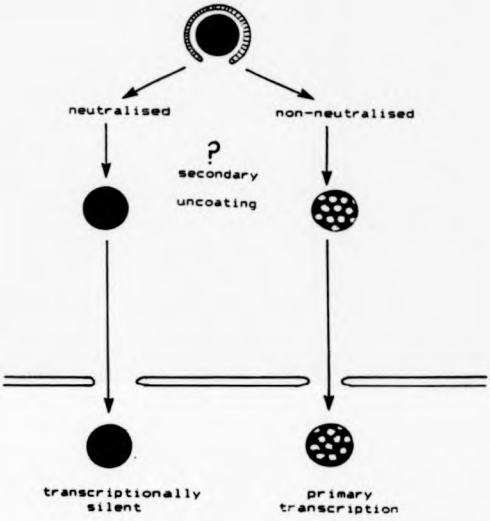


Figure 75: Proposed mechanism of neutralisation of influenza virus by anti-HA IgG.

neutralising IgG attaches to and penetrates cells and undergoes primary uncoating, but not secondary uncoating. Consequently the viral core which becomes localised in the nucleus is transcriptionally inactive. This modified theory explains the data presented in this thesis and those of Possee (1981) and Possee et al. (1982). This mechanism may also be true of other neutralising IgGs, and possibly monomeric IgA, although this remains to be demonstrated, but not secretory IgA or IgM which interfere with attachment and penetration (Taylor and Dimmock, 1985a,b).

A number of examples of antibody-induced conformational changes in proteins, including the influenza virus HA, are known (see General Introduction, Part 16(b)) but their relevance to neutralisation remains obscure. Antibody-induced conformational rearrangements could be detected spectroscopically, by altered sensitivity to proteases and changes in solubility and antigenicity, in the same way that conformational changes occurring in the HA at low pH are detected. The spectroscopic studies of Kida et al. (1985) and Wharton et al. (1986), whilst not actually looking for such a change, provide no evidence for conformational change induced by antibody, but it may be that the putative change is not sufficiently large to be detectable by these methods.

Kida and co-workers have investigated the ability of neutralising IgG and Fab fragments to reduce infectivity and inhibit membrane fusion activity (Kida et al., 1985; Yoden et al., 1985) and their data may provide clues about changes induced in virions by neutralising antibody. Their evidence suggests that bivalent antibody is required for neutralisation, although Fab fragments

derived from monoclonal antibody to two separate sites retained about 2-3% of the neutralising activity of the parent IgGs for the same molar quantities of paratope. Fab fragments from IgGs to the other two sites had no detectable neutralising activity (Yoden et al., 1985). Failure of Fab fragments to reduce neutralisation was paralleled by inability to inhibit haemolysis (Kida et al., 1985). Evidence is presented in Appendix A that Fab' fragments from two anti-H7 monoclonal IgGs (HC2 and HC61) used in this thesis also do not cause neutralisation. There are some grounds for suggesting therefore that bivalency appears to be required for whatever change in virion structure is induced by IgG. If this is a conformational change in the HA then axial rotation of the Fab arms as described by Wrigley et al. (1983) may be required. In this regard it would be interesting to know if the single IgG bound per HA spike under saturating conditions, binds bivalently to one spike or cross-links adjacent spikes (Figure 73, Results and Discussion Section V).

Assuming that anti-HA initiates a transmembrane signal, some means of communication is required. HA is capable of undergoing conformational changes which could allow intramolecular transmission as described for insulin (General Introduction, Part 16(c)). Approximately 11 amino acids comprising the carboxy terminus of HA2 are thought to be exposed on the internal surface of the virion lipid bilayer and could provide the contact with an internal virion component(s), most likely M1 or M2. Attempts have been made to chemically cross-link HA to internal components (N.J.Dimmock and A.Guest, unpublished data). HA-containing heterodimers were detected by Western Blotting using monospecific antibodies, but no differences were seen between infectious and neutralised virus. Recently Taylor et al. (1987) reported

evidence consistent with there being only a small fraction of HA spikes which mediate neutralisation (General Introduction, Part 12) and postulated that the internal component which the neutralisation relevant HA spikes contact is M2. Approximately 20 of the 1,000 HA spikes per influenza virus particle mediate neutralisation and Lamb and co-workers estimate that there are 20-60 molecules of M2 per virion in preparations of purified virus (General Introduction, Part 3(e)). The cross-linking experiments therefore need re-evaluating. Treatment of virus with non-ionic detergent at a suitable concentration leaves some of the HA spikes attached to the virion and more HA remains associated with detergent-produced cores from neutralised virus (Results and Discussion, Section V). It may be that these spikes are more stably associated with the internal virion components and cross-linking would reveal the protein molecules with which these spikes interact. There is evidence that neutralisation of bunyaviruses is dependent on both external and internal proteins. Reassortants with G1 glycoprotein recognised by a neutralising monoclonal antibody are only neutralised if the nucleocapsid protein is from the homologous virus (W.S.James, personal communication). Breschkin et al. (1981) reported the isolation of influenza virus escape mutants which still bind the neutralising monoclonal antibody used for selection. These escape mutants may differ from the parent in the amino acid sequence of the HA, and/or some other virion component, so that neutralisation, but not antibody binding, is abrogated. Sequencing of the genes of viruses with this phenotype therefore may reveal the components involved in the antibody-induced modification of virions.

It has been suggested that neutralising antibody prevents the conformational change in the HA required for membrane fusion

(Kida et al., 1985). The only evidence to support this comes from in vitro studies showing that haemolysis (Kida et al., 1985) and liposome fusion (Wharton et al., 1986) are prevented in the presence of anti-HA IgG. One possibility that this raises, though there is at present no evidence in its favour, is that neutralised virus fails to fuse in endosomes, is transferred to lysosomes and becomes localised in vesicles that co-purify with nuclei during cell fractionation. The viral RNA may then be resistant to RNase because of failure of secondary uncoating or presence in vesicles which are not disrupted by sonication. The evidence from carefully validated cell fractionation studies, however, shows that nuclear accumulation of viral RNA from neutralised virus is indistinguishable from that of infectious virus and that the [^{3}H]-choline label of purified virus remains cytoplasmic (Possee et al., 1982). It is important that these disparate opinions be reconciled by further experiments. The kinetics of uptake suggest that infectious and neutralised influenza virus enter cells by the same pathway. Attachment of infectious and neutralised virus is prevented by treatment of cells with neuraminidase (N.J.Dimmock, unpublished results), suggesting that both use a sialic acid receptor. It is not known if neutralised and infectious virus follow the same endocytotic pathway, although primary uncoating of neutralised virus occurs with normal kinetics (Possee et al., 1982). The pathway could be investigated by analysis of the uptake of neutralised virus by cells in the presence of NH_4Cl or by testing the ability of neutralised virus to cause extracellular fusion of permissive cells.

The evidence for localisation of the viral RNA in the nucleus comes from fractionation of a number of cell types by Dounce

homogenisation and by the nuclear monolayer technique (Possee, 1981; Possee *et al.*, 1982; Dimmock *et al.*, 1984; Taylor, 1986; Section IV, Results and Discussion). These results could be complemented by *in situ* detection of input virion RNA or protein. The use of monoclonal antibodies would yield most information. It would be possible by this method to identify the site of localisation of the core components, the glycoproteins and anti-HA antibody. It may also be possible to investigate secondary uncoating using anti-M and anti-NP monoclonal antibodies if determinants on the NP are masked in M1-RNP, i.e. cores that have not undergone secondary uncoating. It is not known if the cores from infectious and neutralised virus have the identical intranuclear location.

The theory of neutralisation of influenza virus proposed here would be strengthened by direct evidence for failure of neutralised virus to undergo secondary uncoating, possibly by comparing the protein composition of cores extracted from the nuclei of cells inoculated with infectious or neutralised virus. If the effect of neutralising IgG was solely on secondary uncoating one would predict that the transcriptase activity *in vitro* of cores recovered from the nuclei of cells inoculated with neutralised virus could be activated by detergent treatment and would be normal. This might be technically difficult as attempts made to measure primary transcription in the nuclei of cells inoculated with infectious virus (Beaton and Krug, 1984, 1986; del Roi *et al.*, 1985) by incorporation of [^{3}H]-UTP in the presence of inhibitors of cellular enzymes and by dot blot hybridisation were only successful with nuclei extracted from cells which had been inoculated and then incubated at 37°C for 3 hours. By this time presumably enough progeny virus had

accumulated to give detectable quantities of transcripts. However, with a probe of higher specific radioactivity, such as a single-stranded probe labelled throughout its length, it ought to be possible to detect the transcription products of the input virus by hybridisation. Another approach would be to inoculate cells with neutralised or infectious virus, extract endosomes and fuse virus and endosomes *in vitro* by adding ATP. This ought to liberate viral cores and their transcriptase activity and component proteins could be analysed and compared.

Finally, it remains to be demonstrated if IgG to all five antigenic sites on the HA neutralise infectivity by the same mechanism and which of these sites mediate inhibition of transcriptase activity *in vitro* (Possee *et al.*, 1982; Shimizu *et al.*, 1985; see Discussion, Results and Discussion Section II).

Appendix A: Neutralisation fo influenza virus by IgG and fragments of IgG

It is of interest to determine whether monovalent fragments of antibody are able to neutralise infectivity (see General Discussion) . The results shown in Figure 76 support the conclusion (Yoden *et al.*, 1985) that monovalent fragments of neutralising antibodies do not reduce infectivity. IgG was digested with pepsin to $F(ab')_2$ which were then reduced with cysteine (Figure 10, General Introduction). This yielded a mixture of $F(ab')_2$ and Fab' fragments in the ratio of 4:1 (Figure 17, Methods and Optimisations). In the experiment shown in Figure 76 the quantity of $F(ab')_2$ was equivalent to that present in the Fab' preparation and therefore any differences are due to the Fab' fragments. In the absence of rabbit anti-mouse Fab (RAM-Fab), $F(ab')_2$ fragments reduce infectivity to a greater extent than Fab' preparation but in the presence of RAM-Fab the titres were equivalent. Fab' preparations inhibit haemagglutination after dilution and therefore do not dilution dissociate (Figure 72, Results and Discussion, Section V). The discrepancy in the absence of RAM-Fab can be explained if it is postulated that Fab' fragments are unable to neutralise infectivity but compete with the neutralising $F(ab')_2$ fragments. Kinetic evidence using the same $F(ab')_2$ and Fab' preparation support this conclusion (N.J.Dimmock and R.J.Rigg, unpublished results). It should be noted that where RAM-Fab was used it was added after dilution of virus-antibody fragment mixtures providing further evidence that Fab' fragments derived from this IgG (HC6I) do not dilution-dissociate. Similar results were obtained with Fab' preparation from HC2 monoclonal IgG.

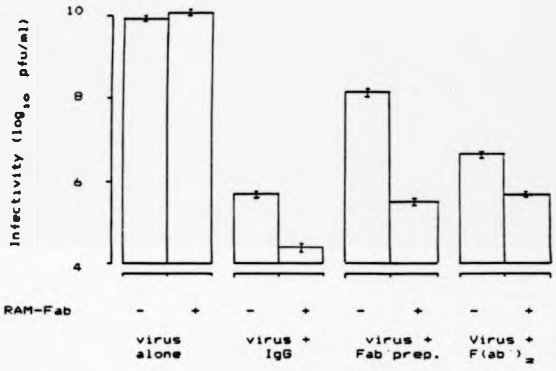


Figure 76: Neutralisation of influenza virus by IgG and antibody fragments, with and without the addition of RAM-Fab after dilution.

REFERENCES

Ada, G.L. and Jones, P.D. 1986. The immune response to influenza infection. Curr. Topics in Microbiol. and Immunol. 128:1.

Alexander, S. and Elder, J.H. 1984. Carbohydrate dramatically influences immune reactivity of antisera to viral glycoprotein antigens. Science 226:1328.

Allen, H., McCauley, J., Waterfield, M., and Gething, M.J. 1980. Influenza virus RNA segment 7 has the coding capacity for two polypeptides. Virol. 107:548.

Almond, J.W. 1977. A single gene determines the host range of influenza virus. Nature (Lond.) 270:617.

Almond, J.W. and Felsenreich, V. 1982. Phosphorylation of the nucleoprotein of an avian influenza virus. J. Gen. Virol. 60:295.

Almond, J.W., McGeoch, D. and Barry, R.D. 1977. Method for assigning temperature-sensitive mutations of influenza viruses to individual segments of the genome. Virol. 81:62.

Aminoff, D. 1961. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. 81:384.

Amit, A.G., Mariuzza, R.A., Phillips, E.V. and Poljak, R.J. 1986. Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution. Science 233:747.

Antczak, J.B., Chmelo, R., Gaillard, R.K., Keene, J.D., Li, J.K.-K., Scheible, P.P. and Joklik, W.K. 1983. Studies on the sequences of reovirus genes (within and among serotypes). In Double-stranded RNA viruses. Compans, R.W. and Bishop, D.H.L. eds. Publ. Elsevier.

Appleyard, G. 1977. Amantadine resistance as a genetic marker for influenza viruses. J.Gen.Virol. 36:249.

Appleyard, G. and Maber, H.B. 1974. Plaque formation by influenza viruses in the presence of trypsin. J. Gen. Virol. 25:351.

Apostolov, K. and Flewett, T.H. 1969. Further observations on the structure of influenza viruses A and C. J.Gen.Virol. 4:365.

Arnheiter, H., Davis, N.L., Wertz, G., Schubert, M. and Lazzarini, R.A. 1985. Role of nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. Cell 41:259.

Ashe, W.K. and Notkins, A.L. 1967. Kinetics of sensitisation of Herpes simplex virus and its relationship to the reduction in the neutralisation rate constant. Virology 33:613.

Atassi, M.Z. 1975. Antigenic structure of myoglobin: the complete immunochemical anatomy of a protein and conclusions relating

to antigenic structures of proteins. Immunochem. 12:423

Avery, R.J. and Dimmock, N.J. 1975. Temporal control of transcription of influenza virus RNA. Virol. 64:409.

Bachi, T., Gerhard, W., Lindenmann, L., and Muhlethaler, K. 1969. Morphogenesis of influenza A viruses in Ehrlich ascites tumour cells as revealed by thin-sectioning and freeze etching. J. Virol. 4:769.

Bachi, T., Gerhard, W. and Yewdell,J.W. 1985. Monoclonal antibodies detect different forms of influenza virus HA during viral penetration and biosynthesis. J. Virol. 55:307.

Barry, R.D., Ives, D.R. and Cruickshank, J.G. 1962. Participation of DNA in the multiplication of influenza virus. Nature (Lond.) 194:1139.

Barrett, T., Wolstenholme, A.J. and Mahy, B.W.J. 1979. Transcription and replication of influenza virus RNA. Virol. 98:211.

Bean, W.J. 1984. Correlation of influenza A virus nucleoprotein with host species. Virol. 133:438.

Bean, W.J., and Simpson, R.W. 1973. Primary transcription of the influenza virus genome in permissive cells. Virol. 56:646.

Beaton, A.R. and Krug, R.M. 1984. Synthesis of templates for influenza virion RNA replication in vitro. Proc. Natl. Acad. Sci., USA 81:4682.

Beaton, A.R. and Krug, R.M. 1986. Transcription anti-termination during influenza viral template RNA synthesis requires the NP protein and the absence of a 5' capped end. Proc. Natl. Acad. Sci., USA 83:6282.

Becht, H., Hammerling, A. and Rott, R. 1971. Undisturbed release of influenza virus in the presence of univalent anti-NA antibody. Virol. 46:337.

Beebe, D.P., Schreiber, R.D. and Cooper, N.R. 1983. Neutralisation of influenza virus by normal human sera: Mechanisms involving antibody and complement. J. Immunol. 130:1317.

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N. Hannum,C., Leach, S.J., Margoliash, E., Micheal, J.G., Miller, A., Prager, E.M. Reichman,M., Sercarz, E.E., Smith-Gill, S.J., Todd, J.P.A. and Wilson, A.C. 1984. The antigenic structure of proteins: A reappraisal. Ann. Rev. Immunol. 2:67.

Beyer, W.E.P., Ruigrok, R.W.H., van Driel, H. and Masurel, N. 1986. Influenza strains with a fusion threshold of pH 5.5 or lower are inhibited by amantadine. Arch. Virol. 90:173.

Blaas, D.B., Patzelt, E., Keuchler, E. 1982a. Cap-recognising protein of influenza virus. Virol. 116:339.

Blaas, D.B., Patzelt, E., Keuchler, E. 1982b. Identification of

the cap-binding protein of influenza virus. Nuc. Acids Res. 10:4803.

Blok, J., Air, G.M., Laver, W.G., Ward, C.W., Lilley, G.S., Woods, E.F., Roxburgh, C.M. and Inglis, A.S.. 1982. Studies on the size, chemical composition, and partial sequence of the neuraminidase (NA) from type A influenza virus show that the N-terminal region of the NA is not processed and serves to anchor the NA in the viral membrane. Virology 119:109.

Boone, L.R. and Skalka, A. 1980. Two species of full-length cDNA are synthesized in high yield by melittin-treated avian retrovirus particles. Proc. Natl. Acad. Sci., USA 77:847.

Bosch, F.X., Orlich, M., Klenk, H-D. and Rott, R. 1979. The structure of haemagglutinin, a determinant for the pathogenicity of influenza virus. Virology 95:197.

Bosch, F.X., Garten, W. Klenk, H-D. and Rott, R. 1981. Proteolytic cleavage of influenza virus haemagglutinins: Primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology 113:725.

Both, G.W., Lavi, S. and Shatkin, A.J. 1975. Synthesis of all the gene products of the reovirus genome in vivo and in vitro. Cell 4:173.

Bouloy, M., Morgan, M.A., Shatkin, A.J. and Krug, R.M. 1979. Cap and internal nucleotides of reovirus mRNA primers are incorporated into influenza viral cRNA during transcription in vitro. J. Virol. 32:895.

Bouloy, M., Plotch, S.J. and Krug, R.M. 1978. Globin mRNAs are primers for the transcription of influenza viral RNA in vitro. 1978. Proc. Natl. Acad. Sci. U.S.A. 75:4886-4890.

Bouloy, M., Plotch, S.J. and Krug, R.M. 1980. Both the 7-methyl and 2'-O-methyl groups in the cap of mRNA strongly influence its ability to act as primer for influenza RNA transcription. Proc. Natl. Acad. Sci., USA 77:3952.

Braam, J., Ulmanen, I. and Krug, R.M. 1983. Molecular model of a eukaryotic transcription complex: Functions and movements of influenza P proteins during capped RNA-primed transcription. Cell. 34:609.

Braam-Markson, J., Jaudon, C. and Krug, R.M. 1985. Expression of a functional influenza viral cap-recognising protein by using a bovine papilloma virus vector. Proc. Natl. Acad. Sci. U.S.A. 82:4326.

Breidis, D.J., Conti, G., Munn, E.A. and Mahy, B.W.J. 1981. Migration of influenza virus-specific polypeptides from cytoplasm to nucleus of infected cells. Virol. 111:154.

Breschkin, A.M., Ahern, J., and White, D.O. 1981. Antigenic determinants of influenza virus HA. VIII. Topography of the antigenic regions of influenza virus determined by competitive radioimmunoassay with monoclonal antibodies. Virol. 113:130.

Breuning, A. and Scholtissek, C. 1986. A reassortant between influenza A viruses (H7N2) synthesizing an enzymatically inactive neuraminidase at 40° which is not incorporated into infectious particles. Virol. 150:65.

Briaen, P., Dekegel, D. and Boeye, A. 1983. Neutralisation of poliovirus by antibody-mediated polymerisation. Virol. 127:463.

Briaen, P., Thomas, A.A.M. and Boeye, A. 1985. Lack of quantitative correlation between the neutralisation of poliovirus and the antibody-mediated pI shift of the virions. J. Gen. Virol. 66:609.

Bubovich, V.I., Ryazantseva, G.M. and Indulen, M.K. 1985. Deproteinisation of influenza virus in the presence of rimantadine. Acta Virol. 29:104.

Bucher, D., Kharitonenkon, J., Zakomirdin, I.G., Grigoriev, B.V., Klimenko, S.M. and Davis, J.F. 1980. Incorporation of influenza M-protein into liposomes. J. Virol. 36:586.

Buchmeier, M.J., Lewicki, H.A., Talbot, P.J. and Knobler, R.L. 1984. Murine hepatitis virus-4 (strain JHM)-induced neurological disease is modulated in vivo by monoclonal antibody. Virol. 132:261.

Buckler-White, A.J., Naeve, C.J. and Murphy, B.R. 1986. Characterisation of a gene coding for M proteins which is involved in host range restriction of an avian influenza virus in monkeys. J.Virol. 57:697.

Bukrinskaya, A., Starov, A. and Issaya, C. 1981. Abortive infection of Ehrlich tumour cells by influenza virus: virus-specific RNA, protein synthesis and transport. Intervirology 16:43.

Bukrinskaya, A.G., Vorkunova, N.K., Kornilayeva, G.V., Narmanbetova, R.A. and Vorkunova, G.K. 1982. Influenza virus uncoating in infected cells and effect of rimantadine. J. Gen. Virol. 60:49.

Bukrinskaya, A.G., Vorkunova, N.K. and Narmanbetova, R.A. 1980. Rimantadine hydrochloride blocks the second step of influenza virus uncoating. Arch. Virol. 66:275.

Burton, D. 1985. Immunoglobulin G: Functional sites. Mol. Immunol. 22:161.

Carroll, S.M., Higa, H.H. and Paulson, J.C. 1981. Different cell surface receptor determinants of antigenically similar influenza virus haemagglutinin. J. Biol. Chem. 256:8357.

Carroll, A.R. and Wagner, R.R. 1979. Role of the membrane (M) protein in endogenous inhibition of in vitro transcription by vesicular stomatitis virus. J. Virol. 29:134.

Carthew, P. 1976. The surface of proteins of a bovine enteroviruses, before and after neutralisation. J. Gen. Virol. 32:17.

Caton, A.J., Brownlee, G.G., Yewdell, J.W. and Gerhard, W. 1982. The antigenic structure of the influenza virus A/PR/8/34 haemagglutinin (H1 subtype). Cell 31:417.

Celada, F., Schumaker, V.N. and Sercarz, E.E. 1983. The changes in protein antigen conformation induced by antibody. In 'Protein Conformation as an immunological signal' eds. as above. Publ. Plenum Press, New York and London. p.211.

Chanas, A.C., Gould, E.A., Clegg, J.C.S. and Varma,M.G.R. 1982. Monoclonal antibodies directed to Sindbis virus glycoprotein E1 can neutralise, enhance infectivity and independently inhibit haemagglutination or haemolysis. J. Gen. Virol. 58:37.

Choppin, P.W., Compans, R.W., Scheid, A., McSharry, J.J. and Lazarowitz, S.G. 1972. In 'Membrane Research'. Fox, J.W. ed. Publ. Academic Press, New York. p.163.

Chothia, C., Lesk, A.M., Dodson, G.G. and Hodgkin,D.C. 1985. Transmission of conformational change in insulin. Nature (Lond.) 302:500.

Clegg, J.C.S., Chanas, A.C., and Gould, E.A. 1983. Conformational changes in Sindbis virus E1 glycoprotein induced by monoclonal antibody binding. J. Gen. Virol. 64:1121.

Colman, P.A., Varghese, J.N. and Laver, W.G. 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature (Lond.) 303:41.

Colman, P.M., Laver, W.G., Varghese, J.N., Baker, A.T., Tulloch, P.A., Air, G.M. and Webster, R.G. 1987. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. Nature (Lond.) 326:358.

Compans, R.W. 1973a. Influenza virus proteins. II. Association with components of the cytoplasm. Virol. 51:56.

Compans, R.W. 1973b. Distinct carbohydrate contents of influenza virus glycoproteins in smooth and rough cytoplasmic membranes. Virol. 55:541.

Compans, R.W. and Choppin, P.W. 1975. In 'Comprehensive virology' vol. 4. Fraenkel-Conrat, H. and Wagner, R.R. eds. Publ. Plenum Press, New York. p.179.

Compans, R.W., Content, J. and Duesberg, P.H. 1972. Structure of the ribonucleoprotein of influenza virus. Virol. 10:795.

Compans, R.W., and Dimmock, N.J. 1969. An electronmicroscopic study of single-cycle infection of chick embryo fibroblasts by influenza virus. Virol. 39: 499.

Compans, R.W., Dimmock, N.J. and Meier-Ewert, H. 1969. Effect of antibody to neuraminidase on the maturation and haemagglutinating activity of an influenza A_2 virus. J. Virol. 4:528.

Compans, R.W., Klenk, H-D., Caliguiri, L.A. and Choppin, P.W.

1970. Influenza virus proteins I. Analysis of polypeptides of the virion and identification of spike glycoproteins. Virol. 42:880.

Cook, R.F., Avery, R.J. and Dimmock, N.J. 1979. Infection of chicken erythrocytes with influenza and other viruses. Inf. and Immun. 25:396.

Cooper, A. 1980. Conformational fluctuation and change in biological macromolecules. Sci. Prog. Oxf. 66:473.

Daniels, C.A. 1975. Mechanisms of viral neutralisation. In 'Viral Immunology and Immunopathology'. Notkins, A.L. ed. Academic Press, New York. p.79.

Daniels, R.S., Douglas, A.R., Skehel, J.J., Wiley, D.C., Naeve, C.W., Webster, R.G., Rogers, G.N. and Paulson, J.C. 1983. Antigenic analysis of influenza virus with different receptor-binding specificities. Virol. 130:174.

Daniels, R.S., Downie, J.C., Hay, A.J. Knossow, M., Skehel, J.J., Wang, M.L. and Wiley, D.C. 1985. Fusion mutants of the influenza virus HA glycoprotein. Cell 40:431.

Daniels, R.S., Jeffries, S., Yates, P., Schild, G.C., Rogers, G.N., Paulson, J.C., Wharton, S.A., Douglas, A.R., Skehel, J.J. and Wiley, D.C. 1987. Receptor-binding and membrane-fusion properties of influenza virus variants selected using anti-HA monoclonal antibody. EMBO J. 6:1459.

Davey, J. 1984. PhD thesis, University of Warwick.

Davey, J., Dimmock, N.J. and Colman, A. 1985. Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in Xenopus oocytes. Cell 40:667.

Davey, J., Hurtley, S.M., and Warren, G. 1985. Reconstitution of an endocytic fusion event in a cell-free system. Cell 43:643.

Davies, J.F and Bucher, D.J. 1981. Interaction of neuraminidase with M-protein in liposomes. In 'Replication of negative strand viruses. Bishop, D.H. and Compans, W. eds. Publ. Elsevier North Holland. p.203.

Davies, D.R. and Metzger, H. 1983. Structural basis of antibody function. Ann. Rev. Immunol. 1:87.

Dawson, C.R., Drake, A.F., Helliwell, J. and Hider, R.C. 1978. The interaction of bee melittin with lipid bilayer membranes. Biochim. et Biophys. Acta 510:75.

Della-Porta, A.J. and E.G. Westaway. 1977. A multi-hit model for the neutralisation of animal viruses. J. Gen. Virol. 38:1.

Deom, C.M., Caton, A.J. and Schulze, I.T. 1986. Host cell-mediated selection of a mutant influenza A virus that has lost a complex oligosaccharide from the tip of the HA. Proc. Natl. Acad. Sci.,USA 83:3771.

Desselberger, U. and Palese, P. 1978. Molecular weights of RNA segments of influenza A and B viruses. Virol. 88:394.

Detjen, M., St.Angelo, C., Katze, M.G. and Krug, R.M. 1987. The three influenza virus polymerase proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. J. Virol. 61:16.

Dimmock, N.J. 1969. New virus specific-antigens in cells infected with influenza virus. Virol. 39. 224.

Dimmock, N.J. 1982. Initial stages in infection with animal viruses. J. Gen. Virol. 59:1.

Dimmock, N.J. 1984. Mechanisms of neutralisation of animal viruses. J. Gen. Virol. 65:1015.

Dimmock, N.J. 1987. Multiple mechanisms of neutralisation of animal viruses. TIBS 12:70.

Dimmock, N.J., Carver, A.S., Kennedy, S.I.T., Lee, M.R. and Luscombe, S. 1977. Internal proteins of influenza virus: ^{35}S-methionine peptide maps as genetic markers. J. Gen Virol. 36:503.

Dimmock, N.J., Taylor, H.P. and Carver, A.S. 1984. Interaction of neutralised influenza virus with avian and mammalian cells. In 'Segmented Negative Strand Viruses'. Compans, R.W.L. and Bishop, D.H.L. eds. Academic Press, New York. p.355.

Dimmock, N.J. and Watson, D.H. 1969. Proteins specified by influenza virus in cells: Analysis by polyacrylamide gel electrophoresis of antigens not present in the virus particle. J. Gen Virol. 5:499.

Doms, R.W. and Helenius, A. 1986. Quaternary structure of influenza virus HA after acid treatment. J. Virol. 60:833.

Doms, R.W., Gething, M.-J., Henneberry, J., White, J. and Helenius, A. 1986. A variant influenza virus HA that induces fusion at elevated pH. J. Virol. 57:603.

Doms, R.W., Helenius, A. and White, J. 1985. Membrane activity of the influenza virus HA: the low-pH induced conformational change. J. Biol. Chem. 260:2973.

Dorrington, K.J. and Klein, M.H. 1982. Binding sites for Fc gamma receptors on immunoglobulin G and factors influencing their expression. Mol. Immunol. 19:1215.

Dourmashkin, R.R., and Tyrrell, D.A.J. 1970. Attachment of two myxoviruses to ciliated epithelial cells. J. Gen. Virol. 9:77.

Doyle, C., Roth, M.G., Sambrook, J. and Gething, M.-J. 1985. Mutations in the cytoplasmic domain of the influenza virus HA affect different stages of intracellular transport. J. Cell Biol. 100:704.

Dowdle, W.R., Downie, J.C. and Laver, W.G. 1974. Inhibition of virus release by antibodies to surface antigens of influenza viruses. J. Virol. 13:269.

Dubois-Dalcq, M., Holmes, K.V. and Rentier, B. 1984. Assembly of enveloped RNA viruses. Publ. Springer-Verlag, Wien, New York.

Duesberg, P.H. 1969. Distinct subunits of the ribonucleoprotein of influenza virus. J. Mol. Biol. 42:485.

Dulbecco, R., Vogt, M. and Strickland, A.G.R. 1956. A study of the basic aspects of neutralisation of two animal viruses, Western equine encephalitis virus and poliomyelitis virus. Virol. 2:162.

Dunn, W.A., Hubbard, A.L. and Aronson, N.N. 1980. Low-temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. J. Biol. Chem. 255:5971.

Elder, K.T., Bye, J.M., Skehel, J.J., Waterfield, M.D. and Smith, A.E. 1979. In vitro synthesis, glycosylation, and membrane insertion of influenza virus haemagglutinin. Virol. 95:345.

Emini, E.A., Kao, S.-Y., Lewis, A.J., Crainic, R. and Wimmer, E. 1983a. Functional basis of poliovirus neutralisation determined with monospecific neutralising antibodies. J. Virol. 46:466.

Emini, E.A., Dorner, A.J., Dorner, L.F., Jameson, B.A. and Wimmer, E. 1983b. Identification of a poliovirus neutralisation epitope through use of neutralising antiserum raised against a purified viral structural protein. Virol. 124:144.

Enami, M., Fukuda, R. and Ishihama, A. 1985. Transcription and replication of 8 RNA segments of influenza virus. Virol. 142:68.

Ennis, F.A. 1982. Some newly recognised aspects of resistance against and recovery from influenza. Arch. Virol. 73:207.

Feinstein, A., Richardson, N.E. and Taussig, M.J.. 1986. Immunoglobulin flexibility in complement activation. Immunol. Today. 7:169.

Fenner, F., McAuslan, B.R., Mims, C.A., Sambrook, J. and White, D.O. 1974. The Biology of Animal viruses. 2nd edition. Academic Press, New York.

Fields, S., Winter, G. and Brownlee, G.G. 1981. Structure of the neuraminidase gene in human influenza A/PR/8/34. Nature (Lond.) 290:213.

Follett, E.A.C., Pringle, C.R., Wunner, W.H. and Skehel, J.J. 1974. Virus replication in enucleate cells: VSV and influenza virus. J. Virol. 13: 394.

Fujinami, R.S., Norrby, E., and Oldstone, M.B.A. 1984. Antigenic modulation induced by monoclonal antibodies: antibodies to measles virus HA alters expression of other viral

polypeptides in infected cells. J. Immunol. 132:2618.

Fuller, S., von Bonsdorf, C.-H. and Simons, K. 1984. VSV infects and matures only through the basolateral surface of the polarised epithelial cell line MDCK. Cell 38:65.

Fuller, A.O. and Spear, P.G. 1985. Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralising antibodies. J. Virol. 55:475.

Furuichi, Y. 1974. "Methylation-coupled" transcription by virus associated transcriptase of cytoplasmic polyhedrosis virus containing double stranded RNA. Nuc. Acids Res. 1:809.

Furuichi, Y. and Shatkin, A.J. 1976. Differential synthesis of blocked and unblocked 5' termini in reovirus mRNA: Effect of pyrophosphate and pyrophosphatase. Proc. Natl. Acad. Sci.,USA 73:3448.

Garoff, H. 1985. Using recombinant DNA techniques to study protein targeting in the eukaryotic cell. Ann. Rev. Biochem. 53:595.

Garten, W. and Klenk, H.D. 1983. Characterisation of the carboxypeptidase involved in the proteolytic cleavage of the influenza HA. J. Gen. Virol. 64:2127.

Garten, W., Linder, D., Rott, R. and Klenk, H.D. 1982. The cleavage site of the HA of fowl plague virus. Virol. 122:186.

Gerhard, W., Yewdell, J., Frankel, M. and Webster, R. 1981. Defining the antigenic structure of an influenza virus HA with hybridoma antibodies and in vitro selected mutants: Implications for antigenic drift. Nature (Lond.) 290:712.

Gething, M-J., and Sambrook, J. 1981. Cell surface expression of influenza haemagglutinin from a cleaved copy of the RNA gene. Nature (Lond.) 293:620.

Gething, M.-J., Doms, R.W., York, D. and White, J. 1986a. Studies on the mechanism of membrane fusion: site-specific mutagenesis of the HA of influenza virus. J. Cell Biol. 102:11.

Gething M.-J., McCammon, K. and Sambrook, J. 1986b. Expression of wild-type and mutant forms of influenza HA: the role of folding in intracellular transport. Cell 46:939.

Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. 1979. Coated pits, coated vesicles and receptor-mediated endocytosis. Nature (Lond.) 279:679.

Gollins, S.W. and Porterfield, J.S. 1984. Flavivirus infection enhancement in macrophages: radioactive and biological studies on the effect of antibody on viral fate. J. Gen. Virol. 65:1261.

Gollins, S.W. and Porterfield, J.S. 1985. Flavivirus infection enhancement in macrophages: An electron microscopy study of viral cellular entry. J. Gen. Virol. 66:1969.

Gollins, S.W. and Porterfield, J.S. 1986a. The uncoating and infectivity of the flavivirus West Nile on interaction with cells: effects of pH and ammonium chloride. J. Gen. Virol.

Gollins, S.W. and Porterfield, J.S. 1986b. A new mechanism for the neutralisation of enveloped viruses by antiviral antibody. Nature (Lond.) 321:244.

Gottschalk, A. 1957. The specific enzyme of influenza virus and Vibrio cholerae. Biochem. Biophys. Acta 23:645.

Graves, P.N., Schulman, J.L., Young, J.F. and Palese,, P. 1983. Preparation of influenza virus subviral particles lacking the HA1 subunit of HA: unmasking of cross-reactive determinants. Virol. 126:106.

Grady, L.J. and W. Kinch. 1985. Two monoclonal antibodies against La Crosse virus show host-dependent neutralising activity. J. Gen. Virol. 66:2773.

Greenspan, D., Krystal, M., Nakada, S., Arnheiter, H., Lyles, D.S. and Palese, P. 1985. Expression of influenza virus NS2 non-structural protein in bacteria and localisation of NS2 in infected eukaryotic cells. J. Virol. 54:833.

Gregoriades, A. 1980. Interaction of influenza M protein with viral lipid and phosphatidylcholine vesicles. J. Virol. 36:470.

Gregoriades, A., Christie, T. and Markarian, K. 1984. The membrane protein of influenza virus occurs in two forms and is a phosphoprotein. J. Virol. 49:229.

Gregoriades, A. and Frangione, B. 1981. Insertion of influenza M protein into the viral lipid bilayer and localisation of site insertion. J. Virol. 40:323.

Hackeman, M., Denman, A.M. and Tyrrell, D.A.J. 1974. Inactivation of influenza virus by human lymphocytes. Clin. Exp. Immunol. 16:583.

Hahon, N.J. 1970. Neutralisation of residual infectivity of Venezuelan equine encelphalomyelitis virus by antiserum and the reversibility of the reaction. J. Gen. Virol. 6:361.

Hansen, D.C., Yguerabide, J. and Schumaker, V.N. 1981. Segmental flexibility of immunoglobulin G antibodies in solution: A new interpretation. Biochemistry 20:6842.

Hay, A.J. 1974. Studies on the formation of the influenza virus envelope. Virol. 60:398.

Hay, A.J., Skehel, J.J., McCauley, J. 1982. Characterisation of influenza virus RNA complete transcripts. Virol. 116:517.

Hay, A.J., Lomniczi, B., Bellamy, A.R. and Skehel, J.J. 1977. Transcription of the influenza virus genome. Virol. 83: 337.

Hay, A.J., Kennedy, N.C.T., Skehel, J.J. and Appleyard, G. 1979. The matrix protein gene determines amantadine sensitivity of

influenza viruses. J. Gen. Virol. 42:189.

Hay, A.J. and Skehel, J.J. 1974. Studies on the synthesis of influenza virus proteins. In 'Negative strand Viruses'. Barry, R.D. and Mahy, B.W.J. eds. Publ. Academic Press, New York.

Hay, A.J., Wolstenholme, A.J., Skehel, J.J. and Smith, M.A. 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. 4:3021.

Haywood, A.M., and Boyer, B.P. 1985. Fusion of influenza virus membranes with liposomes at pH 7.5. Proc. Natl. Acad. Sci., USA 82:4611.

Haywood, A.M. and Boyer B.P. 1986. Time and temperature dependence of influenza virus membrane fusion at neutral pH. J. Gen. Virol. 67:2813.

Hegenness, M.H., Scheid, A. and Choppin, P.W. 1980. Conformation of the helical nucleocapsids of paramyxoviruses and VSV: reversible coiling and uncoiling induced by salt concentration. Proc. Natl. Acad. Sci., USA 77:2631.

Heinz, F.X., Mandl, C., Berger, R., Tuma, W. and Kunz, C. 1984. Antibody-induced conformational changes result in enhanced avidity of antibodies to different antigenic sites on the tick-borne encephalitis virus glycoprotein. Virol. 133:25.

Helenius, A., Kartenbeck, A.J., Simons, K. and Fries, E. 1980. On the entry of Semliki forest virus into BHK-21 cells. J. Cell Biol. 84:404.

Herrler, G., Rott, R. Klenk, H-D., Mueller, H-P., Shukla, A.K. and Schauer, R.. 1985. The receptor destroying enzyme of influenza C virus is neuraminate-O-acetylesterase. EMBO J. 4:1503.

Herz, C., Stavnezer, E., Krug, R.M. and Gurney Jnr, T. 1981. Influenza virus, a RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. Cell 26:391.

Higa, H.H., Rogers, R.N. and Paulson, J.C. 1985. Influenza virus haemagglutinins differentiate between receptor determinants bearing N-acetyl, N-glycollyl and N,O-diacetylneuraminic acids. Virol. 144:279.

Hogle, J.M., Chow, M. and Filman, D.J. 1985. Three-dimensional structure of poliovirus at 2.9 A resolution. Science. 229:1358.

Holland, J.J. and Kiehn, E.D. 1970. Influenza virus effects on cell membrane protein. Science 67:202.

Hommel-Berrey, G.A. and Schloemer, R.H. 1986. Inhibition of banzi viral RNA synthesis in BHK-21 cells by banzi virion matrix protein. Interviol. 25:38.

Hood, L., Kronenberg, M. and Hunkapiller, T. 1985. T cell receptors and the immunoglobulin supergene family. Cell 40:225.

Horikoshi, M., Nakayama, M., Yamaoka, N., Furusawa, I. and Shishiyama, J. 1987. Brome mosaic virus coat protein inhibits viral RNA synthesis in vitro. Virol. 158:15.

Horne, R.W., Waterson, A.P., Wildy, P. and Farnham, A.E. 1960. The structure and composition of the myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. Virol. 11:79.

Horisberger, M.A. 1980. The larger P proteins of influenza are composed of one acid and two basic polypeptides. Virol. 107:302.

Huang, R.T.C., Wahn, K., Klenk, H.-D. and Kohama, T. 1980a. Fusion between cell membrane and liposomes containing the glycoproteins of influenza virus. Virol. 104:294.

Huang, R.T.C., Rott, R. Wahn, K. Klenk, H-D. and Kohama, T. 1980b. The function of the neuraminidase in membrane fusion induced by myxovirus. Virol. 107:313.

Huang, R.T.C., Rott, R. and Klenk, H-D. 1981. Influenza viruses cause haemolysis and fusion of cells. Virol. 110:243.

Huang, R.T.C., Dietsch, E. and Rott, R. 1985. Further studies on the role of neuraminidase and the mechanism of low pH dependence in influenza virus induced membrane fusion. J. Gen. Virol. 66:295.

Huber, R. and Bennett, W.S. 1987. Antibody-antigen flexibility. Nature (Lond.) 326:334.

Hudson, J.B. and Dimmock, N.J. 1977. The use of nuclear monolayers in the study of influenza virus-infected cells. FEMS Microbiol. Lett. 1:325.

Hudson, J.B., Flawith, J. and Dimmock, N.J. 1978. Early events in influenza virus infection. III. The formation of nucleoplasmic RNP complex from the input virion. Virol. 86:167.

Hudson, L. and Hay, F.C. 1980. Practical Immunology. 2nd Edition. Publ. Blackwell, Oxford.

Icenogle, J., Shiwen, H., Duke, G. Gilbert, S., Rueckert, R. and Anderegg, J. 1983. Neutralisation of poliovirus by a monoclonal antibody: Kinetics and stoichiometry. Virol. 127:412.

Inglis, S.C. and Brown, C.M. 1981. Differences in the control of virus mRNA splicing during permissive or abortive infection with influenza A (fowl plague) virus. J. Gen. Virol. 55:153.

Inglis, S.C. and Mahy, B.W.J. 1979. Polypeptides specified by the influenza virus genome. 3. Control of synthesis in infected cells. Virol. 95:154.

Inglis, S.C., Carroll, A.R., Lamb, R.A., and Mahy, B.W.J.. 1976. Polypeptides specified by the influenza virus genome. I.

Evidence for eight distinct gene products specified by fowl plague virus. Virol. 74:489.

Iorio, R.M. and Bratt, M.A. 1984. Neutralisation of NDV by monoclonal antibody to the HN glycoprotein: requirement for antibody to 4 sites for complete neutralisation. J. Virol. 51:445.

Jackson, D.A., Caton, A.J., McCready, S.J. and Cook, P.R. 1982. Influenza virus RNA is synthesized at fixed sites in the nucleus. Nature (Lond.) 296:366.

Jackson, D.C. and Nesterowicz, A. 1985. Antigenic determinants of influenza virus HA. XI. Conformational changes detected by monoclonal antibodies. Virol. 145:72.

Joklik, W. 1964. the intracellular fate of rabbit pox virus rendered non-infectious by various reagents. Virol. 22:620.

Kamata, T. and Watanabe, Y. 1977. Role for nucleocapsid phosphorylation in the transcription of the influenza virus genome. Nature (Lond.) 267:460.

Karadaghi, S.E., Zakomirdin, J.A., Shimane, C., Bucher, D.J., Tversidov, V.A. and Kharitonenko, I.G. 1984. Interaction of influenza virus proteins with planar bilayer lipid membranes. 1. Characterisation of their adsorption and incorporation into lipid bilayers. Biochim. Biophys. Acta 778:269.

Kates, M., Allison, A.C., Tyrrell, D.A.J. and James, A.T. 1961. Lipids of influenza virus and their relation to those of the host cell. Biochem. Biophys. Acta. 52:455.

Kato, A., Mizumoto, K. and Ishihama, A. 1985. Purification and enzymatic properties of an RNA polymerase-RNA complex from influenza virus. Virus Res. 3:115.

Kato, N. and Eggers, H.J. 1969. Inhibition of uncoating of fowl plague virus by 1-adamantanamine hydrochloride. Virol. 37:632.

Katz, J.M., Laver, W.G., White, D.O.and Anders, E.M. 1985. Recognition of influenza virus haemagglutinin by subtype specific and cross-reactive T cells: Contribution of HA1 and HA2 polypeptide chains. J. Immunol. 134:1616.

Kawakami, K., Ishihama, A. and Hamaguchi, M. 1981a. RNA polymerase of influenza virus. I. Comparison of the virion-associated RNA polymerase activity of various strains of influenza virus. J. Biochem. 89:1751.

Kawakami, K., Ishihama, A., Ohtsuka, E., Tanaka, T., Takashima, H. and Ikehara, M. 1981b. RNA polymerase of influenza virus. II. Influence of oligonucleotide chain length on the priming activity of RNA synthesis by virion-associated RNA polymerase. J. Biochem. 89:1759.

Kawakami, K. and A. Ishihama. 1983. RNA polymerase of influenza virus. III. Isolation of RNA polymerase-RNA complexes from influenza PR8. J. Biochem. 93:989.

Kawakami, K., Mizumoto, K., Ishihama, A., Shinozaki-Yamaguchi, K. and Miura, K. 1985. Activation of influenza virus-associated RNA polymerase by Cap 1 structure ($m^7GpppNm$). J. Biochem., 97:655.

Kawaoka, Y.S., Naeve, C.W. and Webster, R.G. 1984. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from HA? Virol. 139:303.

Kelly, D.C., Avery, R.J. and Dimmock, N.J. 1974. Failure of an influenza virus to initiate infection in enucleate BHK cells. J. Virol. 13:1155.

Kelly D.C. and Dimmock, N.J. 1974. Fowl plague virus replication in mammalian cell-erythrocyte heterokaryons: Studies concerning the actinomycin D and ultra-violet sensitive phase in influenza virus replication. Virol. 61:210.

Kemdirim, S., Palefsky, J. and Breidis, D.J. 1986. Influenza B virus PB1 protein: nucleotide sequence of the genome RNA segment predicts a high degree of structural homology with the corresponding influenza A virus polymerase protein. Virol. 152:126.

Kendal, A.P., Galphin, J.C. and Palmer, E.L. 1977. Replication of influenza virus at elevated temperatures: production of virus-like particles with reduced matrix protein content. Virol. 76:186.

Kennedy-Stoskopf, S. and Narayan, O. 1986. Neutralising antibodies to visna lentivirus: mechanism of action and possible role in virus persistence. J. Virol. 59:37.

Kida, H., Yoden, S., Kuwabara, M. and Yanagawa, R. 1985. Interference with a conformational change in the HA molecule of influenza virus by antibodies as a possible neutralisation mechanism. Vaccine 3 suppl.:222.

Kilbourne, E.D., Laver, W.G., Schulman, J.L., and Webster, R.G. 1968. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J. Virol. 2:281.

Kingsbury, D.W. and Webster, R.G. 1969. Some properties of influenza virus nucleocapsids. J. Virol. 4:219.

Kingsbury, D.W. 1985. Orhtomyxo- and Paramyxoviruses and their replication. In 'Virology' Fields, B.N. Ed. in Chief. Publ. Raven Press, New York. p.1157.

Kingsford, L. and Ishizawa, L.D. 1984. Antigenic sites on the G1 glycoprotein of La Crosse virus that are involved in neutralisation. In 'Segmented Negative Strand Viruses'. Compans, R.W.L. and Bishop, D.H.L. eds. Publ. Academic Press, New York p.217.

Kistner, O., Muller, H., Becht, H. and Scholtissek, C. 1985. Phosphopeptide fingerprints of NPs of various influenza A virus strains grown in different host cells. J. Gen. Virol. 66:465.

Kjellen, L. 1985. A hypothesis accounting for the effect of the host cell on neutralisation-resistant virus. J. Gen. Virol. 66:2279.

Kjellen, L., and Schlesinger, R.W. 1959. Influence of host cell on residual infectivity of neutralised vesicular stomatitis virus. Virol. 7:236.

Kjellen, L., and von Zeipel, G. 1984. Influence of host cells on the infectivity and neutralisability of echovirus type 4 (Pesascek). Intervirol. 22:32.

Klenk, H.-D., Garten, W., Bosch, F.X. and Rott, R. 1984. The role of the HA as a determinant for the pathogenicity of avian influenza viruses. In 'The molecular virology and epidemiology of influenza' Eds. Stuart-Harris, C.H. and Potter, C.W. Publ. Academic Press, Orlando, Florida and London. p.193.

Klenk, H.-D., Scholtissek, C. and Rott, R. 1972. Inhibition of glycoprotein biosynthesis of influenza virus by D-glucosamine and 2-deoxy-D-glucose. Virol. 49:723.

Klenk, H-D., Compans, R.W. and Choppin, P.W. 1970. An electronmicroscopic study of the presence or absence of neuraminic acid in enveloped viruses. Virol. 43:1158.

Klenk, H-D., Wollert, W., Rott, R. and Scholtissek, C. 1974. Association of influenza virus proteins with cytoplasmic fractions. Virol. 57:28.

Klenk, H-D., Rott, R. Orlich, M. and Blodorn, J. 1975. Activation of influenza A viruses by trypsin treatment. Virol. 68:426.

Klimov, A.I., Ghendon, Y., Zaudova, M., Broucek, J. and Medveda, T. 1983. High reproduction capacity of recombinants between H3N2 human influenza and fowl plague virus is due to the gene coding for M proteins. Acta Virol. 27:434.

Knossow, M., Daniels, R.S. Douglas, A.R. Skehel, J.J. and Wiley, D.C. 1984. Three dimensional structure of an antigenic mutant of the influenza virus haemagglutinin. Nature (Lond.) 311:678.

Koff,W.C. and Knight, V. 1979. Inhibition of influenza virus uncoating by rimantadine hydrochloride. J. Virol. 31:261.

Korn, R. and Horwitz, M.S. 1986. Adenovirus DNA synthesis *in vitro* is inhibited by the virus-coded major core protein. Virol. 150:342.

Kozeletskaya, K.N., Yukhnova, L.G. and Golubev, D.B. 1984. Characterisation of the effect of rimantadine on intracellular distribution and transport into the nuclei of infected cells of the parent influenza virus genome. Acta Virol. 28:464.

Krug, R.M. 1983. Transcription and replication of influenza viruses. In 'Genetics of influenza viruses'. Palese, P. and Kingsbury, D.W. eds. Publ. Springer-Verlag, Wien, New York. p.70.

Krug , R.M., Bouloy, M. and Plotch, S.J. 1980. RNA primers and the role of host nuclear RNA polymerase II in influenza viral RNA transcription. Phil. Trans. R. Soc. Lond. B. 288:359.

Krug, R.M., Broni, B.A., and Bouloy, M. 1979. Are the 5' ends of influenza viral mRNAs synthesized in vivo donated by host mRNAs? Cell 18:329.

Krug, R.M. and Etkind, P.R. 1973. Cytoplasmic and nuclear virus-specific proteins in influenza virus-infected MDCK cells. Virol. 56:334.

Krug, R.M., Morgan, M.M. and Shatkin, A.J. 1976. Influenza viral mRNA contains internal N^6-methyladenosine and 5' terminal 7-methylguanosine in cap structures. J. Virol. 20:45.

Krug, R.M. and Soisro, R. 1975. Studies on the intracellular localisation of influenza virus-specific proteins. Virol. 64:378.

Krug, R.M., Ueda, M. and Palese, P. 1975. Temperature-sensitive mutants of influenza WSN virus defective in virus-specific RNA synthesis. J. Virol. 16:790.

Kuchino, Y., Nishimura, S., Smith, R.E. and Furuichi, Y. 1982. Homologous terminal sequences in the double-stranded RNA genome segments of CPV of the silkworm Bombyx mori. J. Virol. 44:538.

Lachmann, P.J. 1985. Antibody and complement in viral infections. Brit. Med. Bull. 41:3.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680.

Lafferty, K.J. 1963a. The interaction between virus and antibody. I. Kinetic studies. Virol. 21:161.

Lafferty, K.J. 1963b. The interaction between virus and antibody. II. Mechanisms of the reaction. Virol. 21:176.

Lafferty, K.J. and Gertilis, S. 1963. The interaction between virus and antibody. III. Examination of virus-antibody complexes with the electron microscope. Virol. 21:191.

Lamb, R.A. 1983. The influenza virus RNA segments and their encoded proteins. In 'Genetics of influenza viruses'. Palese, P. and Kingsbury, D.W. eds. Publ. Springer-Verlag, Wien, New York. p.21.

Lamb, R.A., Etkind, P.R. and Choppin, P.W. 1978. Evidence for a ninth influenza viral polypeptide. Virol. 91:60.

Lamb, R.A. and Choppin, P.W. 1977. Synthesis of influenza virus

polypeptides in cells resistant to alpha-amanitin: Evidence for the involvement of cellular RNA polymerase II in virus replication. J. Virol. 23:816.

Lamb, R.A. and Choppin, P.W. 1979. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc. Natl. Acad. Sci.,USA 76:4908.

Lamb, R.A. and Choppin, P.W. 1981. Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. Virol. 112:729.

Lamb, R.A. and Choppin, P.W. 1983. The gene structure and replication of influenza virus. Ann. Rev. Biochem. 52:467.

Lamb, R.A. and Lai, C.-J. 1980. Sequence of interrupted mRNAs and cloned DNA coding for the two overlapping non-structural proteins of influenza virus. Cell 21:475.

Lamb, R.A. and Lai, C.-J. 1982. Spliced and unspliced mRNAs synthesized from cloned influenza virus M DNA in an SV40 vector: Expression of the influenza virus membrane protein (M1). Virol. 123:237.

Lamb, R.A. and Lai, C.-J. 1984. Expression of unspliced NS1 mRNA, spliced NS2 mRNA and a spliced chimera mRNA from cloned influenza virus NS DNA in an SV40 vector. Virol. 135:147.

Lamb, R.A., Lai, C.-J. and Choppin, P.W. 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proc. Natl. Acad. Sci. U.S.A. 78:4170.

Lamb, R.A., Zebedee, S.L. and Richardson, C.D. 1985. Influenza virus M2 is an integral membrane protein expressed on the infected cell surface. Cell 40:627.

Laver, W.G. and Valentine, R.C. 1969. Morphology of the isolated HA and neuraminidase subunits of influenza virus. Virol. 38:105.

Laver, W.G., Air, G.M., Dopheide, T.A. and Ward, C.W. 1980. Amino acid sequence changes in the HA of A/HK (H3N2) influenza virus during the period 1968-1977. Nature (Lond.) 283:454.

Laver, W.G., Air, G.M. and Webster, R.G. 1981. Mechanism of antigenic drift in influenza virus. Amino acid sequence changes in an antigenically active region of Hong Kong (H3N2) influenza virus HA. J. Mol. Biol. 145:339.

Lazarowitz, S.G., Compans, R.W. and Choppin, P.W. 1971. Influenza structural and non-structural proteins in infected cells and their plasma membrane. Virol. 46:830.

Lazarowitz, S.G., Compans, R.W. and Choppin, P.W. 1973a. Proteolytic cleavage of the haemagglutinin polypeptide of the influenza virus: Function of the uncleaved polypeptide HA. Virol. 52:199.

Lazarowitz, S.G., Goldberg, A.R. and Choppin, P.W. 1973b. Proteolytic cleavage by plasmin of the HA polypeptide of

influenza virus: host cell activation of serum plasminogen. Virol. 56:172.

Lazarowitz, S.G. and Choppin, P.W. 1975. Enhancement of infectivity of influenza A and B viruses by proteolytic cleavage of the haemagglutinin polypeptide. Virol. 68:440.

Lazdins, I., Haslam, E.A. and White, D.O. 1972. The polypeptides of influenza virus. VI. Composition of the NA. Virol. 49:758.

Le Comte, J. and Oxford, J.S. 1981. detection of antigenic variation of influenza A matrix protein by a competitive radioimmunoassay. J. Gen. Virol. 57:403.

Lee, P.W.K., Hayes, E.C. and Joklik, W.K. 1981 Protein σ1 is the reovirus attachment protein. Virol. 108:156.

Lefrancois, L. and Lyles, D.S. 1982. The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. II. Monoclonal antibodies to non-neutralising and cross reactive epitopes of Indiana and New Jersey serotypes. Virol. 121:168.

Lenard, J. 1978. Virus envelopes and plasma membranes. Ann. Rev. Biophys. Bioengineering 7:139.

Lenard, J. and Compans, R.W. 1974. The membrane structure of lipid containing viruses. Biochim. Biophys. Acta 344:51.

Lenard, J., Wong, C.Y. and Compans, R.W. 1974. Association of the internal membrane proteins with the lipid bilayer in influenza virus. Biochim. Biophys. Acta 332:341.

Lenard, J. and Miller, D.K. 1983. Entry of enveloped viruses into cells. 1983. In Receptors and recognition, series B Vol. 15. P. Cuatrecasa and T.F. Roth, eds. Chapman and Hall. London. P.120.

Lewenton-Kriss, S. and Mandel, B. 1972 Studies on the non-neutralisable fraction of poliovirus. Virol. 48:666

Lohmeyer, J. and Klenk, H.-D. 1979. A mutant of influenza virus with a temperature sensitive defect in the post-translational processing of the HA. Virol. 93:134.

Lohmeyer, J., Talens, L.T. and Klenk, H.D. 1979. Biosynthesis of the influenza virus envelope in abortive infection. J. Gen. Virol. 42:73.

Lubeck, M.D., Schulman, J.L. and Palese, P. 1978. Susceptibility of influenza A viruses to amantadine is influenced by the gene coding for M protein. J. Virol. 28:710.

Lubeck, M. and Gerhard, W. 1981. Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecule are induced by the binding of monoclonal antibodies. Virol. 118:1.

Maeda, T. and Ohnishi, S. 1980. Activation of influenza virus by acidic media causes haemolysis and fusion of erythrocytes. FEBS Lett. 122:283.

Maeda, T., Kawasaki, K. and Ohnishi, S. 1981. Interaction of influenza virus HA with target membrane lipids is a key step in virus-induced haemolysis and fusion at pH 5.2. Proc. Natl. Acad. Sci., USA 78:4133.

Maeno, K., Yoshii, S., Mita, K., Hamaguchi, M., Yoshida, T., Iinuma, M., Nagai, Y. and Makumoto, T. 1979. Analysis of the inhibitory effect of canavanine on the replication of influenza RA/5+ virus. Virol. 94:128.

Maeno, K., Aoki, H., Tsurumi, T., Tsukeura, S., Shibata, M., Hamaguchi, M., Nagai, Y. and Sugiura, Y. 1981. Analysis of the inhibitory effect of canavanine on the replication of influenza RI/5+ virus: Interaction of M protein with the plasma membrane. Microbiol. Immunol. 25:1279.

Mahy, B.W.J. 1983. Mutants of influenza virus. In 'Genetics of influenza viruses' Palese, P. and Kingsbury, D.W. eds. Publ. Springer-Verlag, Wien and New York. p.192.

Mahy, B.W.J., Barrett, T., Breidis, D.J., Brownson, J.M. and Wolstenholme, A.J. 1980. Influence of the host cell on influenza virus replication. Phil. Trans. R. Soc. (Lond.) B 288:349.

Mandel, B. 1967a. The interaction of neutralised poliovirus with HeLa cells I. Adsorption. Virol. 31:238.

Mandel, B. 1967b. The interaction of neutralised poliovirus with HeLa cells II. Elution, penetration uncoating. Virol. 31:248.

Mandel, B. 1976. Neutralisation of poliovirus: a hypothesis to explain the mechanism and one-hit character of the neutralisation reaction. Virol. 69:500.

Mandel, B. 1979. Interaction of viruses with neutralising antibodies.In Comprehensive Virology. vol.16. Fraenkel-Conrat, H. and Wagner, R.R. eds. Publ. Plenum Press, New York. p.31.

Mandel, B. 1984. Mechanisms of virus neutralisation. In 'Concepts in viral pathogenesis'. Notkins, A.L. and Oldstone, M.B.A. eds. Publ. Springer-Verlag, Wien and New York. p.32.

Mandel, B. 1985. Virus neutralisation. In 'Immunochemistry of viruses'. van Regenmortel, M.V.H. and Neurath, A.R. eds. Publ. Elsevier, Amsterdam, New York and Oxford. p. 53.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. Molecular cloning. A laboratory manual. Publ. Cold Spring Harbor Laboratory, New York.

Mark, G.M., Taylor, J.M., Broni, B. and Krug, R.M. 1979. Nuclear accumulation of influenza viral RNA transcripts and the effects of cycloheximide, actinomycin D and alpha-amanitin. J. Virol. 29:744.

Marsh, M., Bolzau, E. and Helenius, A. 1983. Penetration of Semliki Forest virus from acid prelysosomal vacuoles. Cell. 32:931.

Marsh, M. 1983. Entry of enveloped viruses into cells by endocytosis. Biochem. J. 218:1.

Marsh, M., Griffiths, G., Dean, G.E., Mellman, I. and Helenius, A. 1986. Three-dimensional structure of endosomes in BHK-21 cells. Proc. Natl. Acad. Sci.,USA 83:2899.

Massab, H.F. and DeBorde, D.C. 1983. Characterisation of an influenza A host range mutant. Virol. 130:342.

Massey, R.J. and Schochetman, G. 1981. Viral epitopes and monoclonal antibodies: Isolation of blocking antibodies that inhibit viral neutralisation. Science 213:447.

Mark, G.E., Taylor, J.M., Broni, B. and Krug, R.M. 1979. Nuclear accumulation of influenza viral RNA transcripts and the effects of cycloheximide, Actinomycin D and α-amanitin. J. Virol. 29:744.

Matlin, K.S., Reggio, H., Helenius, A. and Simons, K. 1981. Infectious pathway of influenza virus in a canine kidney cell line. J. Cell Biol. 93:601.

McCauley, J. and Mahy, B.W.J. 1983. Structure and function of the influenza virus genome. Biochem. J. 211:281.

McCauley, J.W., Mahy, B.W.J. and Inglis, S.C. 1982. Nucleotide sequence of FPV RNA segment 7. J. Gen. Virol. 58:211.

McCauley, J., Skehel, J., Elder, K., Gething, M.-J., Smith, A. and Waterfield, M. 1980. HA biosynthesis. In 'Structure and variation in influenza viruses'. Laver, G. and Air, G. eds. Publ. Elsevier North Holland, New York. p.97.

McCrae, M.A. 1985. Double-stranded RNA viruses. In 'Virology, a practical approach'. Mahy, B.W.J. ed. Publ. IRL Press, Oxford and Washington. p.151.

McGeoch, D.J. and Kitron, N. 1975. Influenza virion RNA-dependent RNA polymerase: Stimulation by guanosine and related compounds. J. Virol. 15:686.

Meier-Ewert, H. and Compans, R.W. 1974. Time course of synthesis and assembly of influenza virus proteins. J. Virol. 14:1083.

Mertens, P.C. 1979. Ph.D. thesis, University of Oxford.

Metzger, H. 1970. Structure and function of gamma M macroglobulins. Adv. Immunol. 12:57.

Mikheyeva, A.V. and Ghendon, Y.Z. 1983. In vitro inhibition of negative strand virus transcriptase activity by proteins soluble in acidic chloroform-methanol. J. Gen. Virol. 64:305.

Mims, C.A. and White, D.O. 1984. Viral Pathogenesis and Immunology. Publ. Blackwell. London.

Minor, P.D. and Dimmock, N.J. 1975. Inhibition of synthesis of influenza virus proteins: Evidence for two host-cell dependent events during multiplication. Virol. 67:114.

Minor, P.D. and Dimmock, N.J. 1977. Selective inhibition of influenza virus protein synthesis by inhibitors of DNA function. Virol. 78:393.

Mitchell, D.M., McMichael, A.J. and Lamb, J.R. 1985. The immunology of influenza. Brit. Med. Bull. 41:80.

Miura, K., Fujii, I., Sakaki, T., Fuke, M. and Kawase, S. 1968. Double-stranded RNA from CPV of silkworm. J. Virol. 2:1211.

Mohier, E., Pinck, L. and Hirth, L. 1974. Replication of alfalfa mosaic virus RNAs. Virol. 58:9.

Moller, N.P.H. 1979. Fc mediated immune precipitation. I. A new role of the Fc -portion of IgG. Immunology 38:631.

Moller, N.P.H. and Steensgaard, J. 1979. Fc-mediated immunoprecipitation. II. Analysis of precipitating immune complexes by rate-zonal ultracentrifugation. Immunology 38:641.

Morongiello, M.P. and Dales, S. 1977. Characterisation of cytoplasmic inclusions formed during influenza/ WSN virus infection of CEF cells. Intervirology 8:281.

Morser, M.J., Kennedy, S.I.T., and D.C. Burke. 1973. Virus-specified polypeptides in cells infected with Semliki Forest virus. J. Gen. Virol. 21:19.

Murphy, B.R. and Webster, R.G. 1985. Influenza viruses. In 'Virology'. Fields, B.N. Ed. in Chief. Publ. Raven Press, New York. p.1179.

Murti, K.G., Bean, W.J. Jr., and Webster, R.G. 1980. Helical ribonucleoproteins of influenza virus. An electron microscopic analysis. Virol. 104:224.

Murti, K.G. and Webster, R.G. 1986. Distribution of haemagglutinin and neuraminidase on influenza virions as revealed by immunoelectron microscopy. Virol. 149:36.

Naeve, C.W., Hinshaw, V.S., Webster, R.G. 1984. Mutations in the HA receptor binding site can change the biological properties of an influenza virus. J. Virol. 51:567.

Natali, A., Oxford, J.S. and Schild, G.C. 1981. Frequency of naturally occurring antibody to influenza virus antigenic variants selected in vitro with monoclonal antibody. J. Hyg. Camb. 87:185.

Nermut, M.V. and Frank, H. 1971. Fine structure of influenza A_2 (Singapore) as revealed by negative staining, freeze drying and freeze etching. J. Gen. Virol. 10:37.

Nestorowicz, A., Laver, G. and Jackson, D.C. 1985. Antigenic determinants of influenza virus haemagglutinin. X. A comparison of the physical and antigenic properties of monomeric and trimeric forms. J. Gen. Virol. 66:1687.

Nguyen, T.D., Bottreau, E., Bernard, S., Lantier, I. and Aynaud, J.M. 1986. Neutralising secretory IgA and IgG do not inhibit attachment of transmissible gastroenteritis virus. J. Gen. Virol. 67:939.

Nisonoff, A., Hopper, J.E. and Spring, S.B. 1975. The antibody molecule. Academic Press, New York.

Nisonoff, A. and Pressman, D. 1958. Loss of precipitating activity of antibody without destruction of binding sites. Science 128:659.

Oi, V.T., Vuong, T.M., Hardy, R., Reidler, J., Dangl, J., Herzenberg, L.A. and Stryer, L. 1984. Correlation between segmental flexibility and effector functions of antibodies. Nature (Lond.) 307:136.

Ortin, J., Martinez, C., del Rio, L., Davila, M., Lopez-Galindez, C., Vilandeva, N. and Domingo, E. 1983. Evolution of the nucleotide sequence of influenza virus RNA segment 7 during drift of the H3N2 subtype. Gene 23:233.

Pal, R., Grinnell, B.W., Snyder, R.M. and Wagner, R.R. 1985. Regulation of viral transcription by the matrix protein of VSV probed by monoclonal antibody and t.s. mutants. J. Virol. 56:386.

Palese, P. and Schulman, J.L. 1974. Isolation and characterisation of influenza virus recombinants with high and low neuraminidase activity. Use of 2-(3'-methoxyphenyl)-N-acetylneuraminic acid to indentify cloned populations. Virol. 57:227.

Palese, P., Tobita, K., Ueda, M. and Compans, R.W. 1974. Characterisation of temperature sensitive influenza virus mutants defective in neuraminidase. Virol. 61:397.

Palese, P., Ritchey, M.B. and Schulman, J.L. 1977. P1 and P3 proteins of influenza virus are required for complementary RNA synthesis. J. Virol. 21:1187.

Patterson, S. and Oxford, J.S. 1986. Early interactions between animal viruses and the host cell: relevance to viral vaccines. Vaccine 4:79.

Patterson, S., Oxford, J.S. and Dourmashkin, R.R. 1979. Studies on the mechanism of influenza virus entry into cells. J. Gen. Virol. 43:223.

Pattnaik, A.K., Brown, D.J. and Nayak, D.P. 1986. Formation of influenza virus particles lacking HA on the viral envelope. J. Virol. 60:994.

Paulson, J.C., Sadler, J.E. and Hill, R.L. 1979. Restoration of specific myxovirus receptors to asialoerythrocytes by incorporation of sialic acid with pure sialotransferases. J. Biol. Chem. 254:2120.

Payne, C.C. and Mertens, P.C. 1983. Cytoplasmic polyhedrosis viruses. In The reoviridae'. Joklik, W.K. ed. Publ. Plenum

Press, New York and London. p.425.

Pecht, I., Givol, D. and Sela, M. 1972. Dynamics of hapten-antibody interaction. Studies on a myeloma protein with anti -2,4-Dinitrophenyl specificity. J. Mol. Biol. 68:241.

Pedley, C.B. and Cooper, R.J. 1987. The assay, purification and properties of vaccinina virus-induced uncoating protein. J. Gen. Virol. 68:1021.

Peiris, J.S.M., Porterfield, J.S. and Roehrig, J.T. 1982. Monoclonal antibodies against the flavivirus West Nile. J. Gen. Virol. 58:283.

Penn, C.R., Blaas, D., Kuechler, E. and Mahy, B.W.J. 1982. Identification of the cap binding protein of two strains of Influenza A/FPV. J. Gen. Virol. 62:177.

Penn, C.R. and Mahy, B.W.J. 1984. Capped mRNAs may stimulate the influenza virion polymerase by allosteric modulation. Virus Res. 1:1.

Peterson, O.W. and van Deurs, B. 1983. Serial-section analysis of coated pits and vesicles involved in adsorptive pinocytosis in cultured fibroblasts. J. Cell Biol. 96:277.

Petri, T. and Dimmock, N.J. 1981. Phosphorylation of influenza virus nucleoprotein *invivo*. J. Gen. Virol. 57:185.

Petri, T., Patterson, S. and Dimmock, N.J. 1982. Polymorphism of the NS1 proteins of type A influenza virus. J. Gen. Virol. 61:217.

Phillipson, L. 1966. Interaction between poliovirus and immunoglobulins. II. Basic aspects of a virus-antibody interaction. Virol. 28:35.

Plotch, S.J. and Krug, R.M. 1977. Influenza virion transcriptase: Synthesis *in vitro* of large, polyadenylic acid containing complementary RNA. J. Virol. 21:24.

Plotch, S.J. and Krug, R.M. 1978. Segments of influenza virus complementary RNA synthesized *in vitro*. J. Virol. 25:579.

Plotch, S.J., Tamasz, J. and Krug, R.M. 1978. Absence of detectable capping and methylating enzymes in influenza virions. J. Virol. 28:75.

Plotch, S.J., Bouloy, M., Ulmanen, I. and Krug, R.M. 1981. A unique cap (m^7 GpppXm)-dependent influenza virion endonuclease cleaves capped mRNAs to generate the primers that initiate viral mRNA transcription. Cell 23:847.

Pons, M.W. 1972. Studies on the replication of influenza virus RNA. Virol. 47:823.

Pons, M.W., Schulze, I.T. and Hirst, G.K. 1969. Isolation and characterisation of the ribonucleoprotein of influenza virus. Virol. 39:250.

Porter, R.R. 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochem. J. 73:119.

Possee, R.D. 1981. The mechanism of the neutralisation of influenza virus by antibody. Ph.D thesis, University of Warwick.

Possee, R.D. and Dimmock, N.J. 1981. Neutralisation of influenza virus by antibody: Attachment, uptake and uncoating of neutralised virus in chick embryo cells. In "Genetic Variation Among Influenza Viruses" ICN-UCLA Symposium XXI. Nayak D.P. and Fox, C.F. eds. Publ. Academic Press, London. p.473.

Possee, R.D., Schild, G.C. and Dimmock, N.J. 1982. Studies on the mechanism of neutralisation of influenza virus by antibody: Evidence that neutralising antibody (anti-HA) inactivates in influenza virus in vivo by inhibiting virion transcriptase activity. J. Gen. Virol. 58:373.

Poste, G. and Pasternak, C.A. 1978. Virus induced fusion. In Cell Surface Reviews. Vol.5. Poste, G. and Nicholson, G.L. eds. Publ. North Holland Publishing Co., Amsterdam. p.305.

Potter, C.W. and Oxford, J.S. 1979. Determinants of immunity to influenza infection in man. Brit. Med. Bull. 35:69.

Privalsky, M.L. and Penhoet, E.E. 1977. Phosphorylated protein component present in influenza virions. J. Virol. 24:401.

Privalsky, M.L. and Penhoet, E.E. 1978. Influenza virus proteins: Identity, synthesis and modification analysed by two-dimensional electrophoresis. Proc. Natl. Acad. Sci.,USA 75:3625.

Putnam, F.W., Tan, M., Lynn, L.T., Easley, C.W. and Migita, S. 1962. The cleavage of rabbit γ-globulin by papain. J. Biol. Chem. 237:717.

Racaniello, V.R. and Palese, P. 1979. Isolation of influenza C virus recombinants. J. Virol. 32:1006.

Rees P.J. and N.J. Dimmock. 1981. Electrophoretic separation of influenza virus ribonucleoproteins. J. Gen. Virol. 53:125.

Reginster, M. and Nermut, M.V. 1976. Preparation and characterisation of influenza virus cores. J. Gen. Virol. 1976.

Richman, D.D., Hostetler, K.Y., Yazaki, P.J. and Clark, S. 1986. Fate of influenza virion proteins after entry into subcellular fractions of LLC cells and the effect of amantadine. Virol. 151:200.

Rifkin, D.B., Compans, R.W. and Reich, E. 1972. A specific labelling procedure for proteins on the outer surface of membranes. J. Biol. Chem. 247:6432.

Robertson, J.S., Schubert, M. and Lazarini, R.A. 1981. Polyadenylation sites for influenza virus mRNA. J. Virol. 38:157.

Robertson, J.S., Robertson, M.E.St.C. and Roditi, I.J. 1984. Nucleotide sequence of RNA segment 3 of the avian influenza A/FPV/Rostock/34 and its comparison with the corresponding segment of human strains A/PR/8/34 and A/NT/60/68. Virus Res. 1:73.

Roditi, I.J. and Robertson, J.S. 1984. Nucleotide sequence of the avian influenza virus A/FP/Rostock/34 segment 1 encoding the PB2 polypeptide. Virus Res. 1:65.

Rodriguez-Boulan, E.J. and Pendergast, M. 1980. Polarised distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. Cell 20:45.

Rodriguez-Boulan, E. and Sabatini, D.D. 1978. Asymetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. Proc. Natl. Acad. Sci.,USA 75:5071.

Roehrig, J.T. and Mathews, H. 1986. Mechanisms of in vitro neutralisation of alphaviruses by monoclonal antibodies. Abstract: positive strand meeting, Colorado, Easter, 1986.

Rogers, G.N., Daniels, R.S., Skehel, J.J., Wiley, D.C., Wang, X., Higa, H.H. and Paulson, J.C. 1985. Host-mediated selection of influenza virus receptor variants. J. Cell Biol. 260:7362.

Rogers, G.N. and Paulson, J.C. 1983. Receptor determinants of human and animal virus isolates: difference in receptor binding specificity of the H3 HA based on the species of origin. Virol. 127:361.

Rogers, G.N., Paulson, J.C., Daniels, R.S., Skehel, J.J., Wilson, I.A. and Wiley, D.C. 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature (Lond.). 304:76.

del Roi, L., Martinez, C., Domingo, E. and Ortin, J. 1985. In vitro synthesis of full-length influenza virus cRNA. EMBO J. 4:243.

Romanos, M.A. and Hay, A.J. 1984. Identification of the influenza virus transcriptase by affinity-labelling with pyridoxal 5'-phosphate. Virol. 132:110.

Rossman, M.G., Arnold, E., Erickson, J.W., Frankenberger, E.A., Griffith, J.P., Hecht, H.-J., Johnson, J.E., Kramer, G., Luo, M., Mosser, A.G., Rueckert, R.R., Sherry, B. and Vriend, G. 1985. Structure of a common cold virus and functional relationship to other picornaviruses. Nature (Lond.) 317:145.

Rott, R. 1979. Molecular basis of infectivity and pathogenicity of myxovirus. Arch. Virol. 59:285.

Rott, R. and Klenk, H.-D. 1977. Structure and assembly of viral envelopes. In Cell surface reviews'. Poste, G. and Nicholson, G.L. eds. Publ. North Holland, Amsterdam. p.47.

Rott, R. and Klenk, H.-D. 1986. Pathogenicity of influenza virus in model systems. In 'Options for control of influenza virus'. Kendal, A.P and Patriarca, P.A.. Publ. Alan R. Liss,

New York. p.53.

Rott, R., Orlich, M., Klenk, H.-D., Wang, M.L., Skehel, J.J. and Wiley, D.C. 1984. Studies on the adaptation of influenza viruses to MDCK cells. EMBO J. 3:3329.

Rott, R., Reinacher, M., Orlich, M., Klenk, H.-D. 1980. Cleavability of HA determines spread of avian influenza viruses in the chorioallantoic membrane. Arch. Virol. 65:123.

Rott, R. and Scholtissek, C. 1970. Specific inhibition of influenza replication by ε-amanitin. Nature (Lond.) 228:56.

Rubin, H. and Franklin, R.M. 1957. On the mechanism of Newcastle disease virus neutralisation by immune serum. Virol. 3:84.

Rueckert, R.R. 1976. On the structure and morphogenesis of picornaviruses. In 'Comprehensive Virology' Vol. 6. Fraenkel-Conrat and Wagner, R.R. eds. Publ. Plenum Press, New York. p.131.

Ruigrok, R.W.H., Wrigley, N.G., Calder, L.J., Cusack, S., Wharton, S.A., Brown, E.B. and Skehel, J.J. 1986a. Electron microscopy of the low pH structure of influenza virus HA. EMBO J. 5:41.

Ruigrok, R.W.H., Martin, S.R., Wharton, S.A., Skehel, J.J., Bayley, P.M. and Wiley, D.C. 1986b. Conformational changes in the HA of influenza virus which accompany heat-induced fusion of virus with liposomes. Virol. 155:484.

Russell, P.H., Griffiths, P.C., Goswami, K.K.A., Alexander, D.J., Cannon, M.J. and Russell, W.C. 1983. The characterisation of monoclonal antibodies to Newcastle disease virus. J. Gen. Virol. 64:2069.

Russell, P.H. 1986. The synergistic neutralisation of Newcastle disease virus by two monoclonal antibodies to its haemagglutinin-neuraminidase protein. Arch. Virol. 90:135.

Sanger, F. and Coulson, A.R. 1978. The use of thin acrylamide gels for DNA sequencing. FEBS Lett. 87:107.

Sato, S.B., Kawasaki, K. and Ohnishi, S.-I. 1983. Haemolytic activity of influenza virus HA glycoproteins cultivated in mildly acidic environments.Proc. Natl. Acad. Sci., USA 80:3153.

Schauer, R. 1983. Glycosidases with special reference to the pathophysiological role of sialidases. In 'Structural carbohydrates in the liver'. Popper, H., Reutter, W., Kottgen, E. and Gndat, F. Publ. MTP Press, Boston. p.83.

Scheid, A. and Choppin, P.W. 1977. Two disulphide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virol. 80:54.

Schild, G.C., Oxford, J.S. and Newman, R.W. 1979. Evidence for antigenic variation in influenza A nucleoprotein. Virol. 93:569.

Schild, G.C., Oxford, J.S. and Webster, R.G. 1984. Evidence for host cell selection of antigenic variants of influenza A (H1N1) and B virus: Antigenic differences between the haemagglutinins of viruses grown in eggs and mammalian cells. In 'The Molecular Virolgy and Epidemiology of Influenza'. Stuart-Harris, C.H. and Porter, C.W. eds. Publ. Academic Press, London. p.164.

Schild, G.C., Oxford, J.S. and Newman, R.W. 1979. Evidence for antigenic variation in influenza A nucleoprotein. Virol. 93:569.

Schlegel, R. and Wade, M. 1983. Neutralised virus binds to host cells by a different receptor. Biochem. Biophys. Res. Commun. 144:774.

Scholtissek, C. 1986. Influenza A viruses with non-cleaved HA are not internalised after adsorption. Arch. Virol. 90:159.

Scholtissek, C. and Bowles, A.L. 1975. Isolation and characterisation of temperature-sensitive mutants of fowl plague virus. Virol. 67:576.

Schulman, J.L. and Palese, P. 1977. Virulence factors of influenza viruses. WSN virus neuraminidase is required for productive infection of MDCK cells. J. Virol. 24:170.

Schulze, I.T. 1972. The structure of influenza virus. II. A model based on the morphology and composition of subviral particles. Virol. 47:181.

Seto, J.T. and Rott, R. 1966. Functional significance of sialidase during influenza virus multiplication. Virol. 30:731.

Shapiro, G.I., Gurney-Jr, T. and Krug, R.M. 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. J. Virol. 61:764.

Shaw, M.W. and Compans, R.W. 1978. Isolation and characterisation of cytoplasmic inclusions from influenza A virus infected cells. J. Virol. 25:608.

Shibata, M., Maeno, K., Tsurumi, T., Aoki, H., Nishiyama, Y., Ito, Y., Isomura, S. and Suzuki, S. 1982. Role of viral glycoproteins in haemolysis by influenza B viruses. J.Gen. Virol. 59:183.

Shen, L. and Ginsberg, V. 1968. In 'Biological properties of the mammalian surface membrane'. Mosser, P. ed. Publ. Wistar Institute, Philadelphia. p.67.

Shimizu, K. and Ishada, N. 1975. The smallest protein of sendai virus: its candidate function of binding nucleocapsid to envelope. Virol. 67:427.

Shimizu K., Mukaigawa, J., Oguro, M., Ono, Y., Nakajuna, K. and Kida, H. 1985. Inhibition of transcriptase activity of influenza A virus in vitro by anti-HA antibody. Vaccine

3:207.

Silverstein, S.C. and Marcus, P.I. 1964. Early stages of Newcastle Disease Virus-HeLa cell interaction. An electron microscopic study. Virol. 23:370.

Skehel, J.J. 1972. The polypeptide composition of influenza A viruses. Virol. 49:23.

Skehel, J.J. and Schild, G.C. 1971. The polypeptide composition of influenza A viruses. Virol. 44:396.

Skehel, J.J. and Waterfield, M.D. 1975. Studies on the primary structure of the HA of influenza virus. Proc. Natl. Acad. Sci. U.S.A.72:93.

Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D., White, J.M., Wilson, I.A. and Wiley, D.C. 1982. Changes in the conformation of influenza virus HA at the pH optimum of virus mediated membrane fusion. Proc. Natl. Acad. Sci. U.S.A. 79:968.

Skehel, J.J. and Schild, G.C. 1971. The polypeptide composition of influenza A viruses. Virol. 44:396.

Skehel, J.J., Stevens, D.J., Daniels, R.S., Knossow, M., Wilson, I.A. and Wiley, D.C. 1984. A carbohydrate side chain on HAs of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci., USA 81:1779.

Sklyanskaya, E.I., Varich, N.L., Amvrosieva, T.V. and Kaverin, N.V. 1985. Virions and intracellular nucleocapsids produced during mixed heterotypic influenza infection of MDCK cells. J. Virol. 53:679.

Slepushkin, V.A., Bukrinskaya, A.G., Prokazova, N.V., Zhigis, L.S., Reshov, P.D., Shaposhnikova, J.I. and Bergelson, L.S. 1985. Action of influenza virus neuraminidase on gangliosides: HA inhibits viral neuraminidase. FEBS Lett. 182:273.

Smith, R.E. and Furuichi, Y. 1980. Gene mapping of cytoplasmic polyhedrosis virus of silkworm by full-length mRNA prepared under optimised condition of transcription in vitro. Virol. 103:279.

Smith, G.L. and Hay, A.J. 1982. Replication of the influenza genome. Virol. 118:96.

Smith, D.B. and Inglis, S.C. 1985. Regulated production of an influenza virus spliced mRNA mediated by virus-specific products. EMBO J. 4:2313

Spooner, L.L.R. and Barry, R.D. 1977. Participation of DNA-dependent RNA polymerase II in replication of influenza viruses. Nature (Lond.) 268:650.

Stanley, P., Gandhi, S.S. and White, D.O. 1973. The polypeptides of influenza virus. VII. Synthesis of the HA.

Stanley, P.M. and Haslam, E.A. 1971. The polypeptides of

influenza virus. V. Localisation of polypeptides by iodination techniques. Virol. 46:764.

Steensgaard, J. 1984. The mechanism of immune precipitation. Immunology Today 5:7.

Stephenson, J.R. and Dimmock, N.J. 1975. Early events in influenza virus multiplication. I. Location and fate of the input RNA. Virol. 65:77.

Stephenson, J.R., Hudson, J.B. and Dimmock, N.J. 1978. Early events in influenza virus multiplication. II. Penetration of virus into cells at 4°C. Virol. 86:264.

Stridh, S., Oberg, B., Chattopadhyaya, J. and Josephson, S. 1981. Functional analysis of influenza RNA polymerase activity by the use of caps, oligonucleotides and polynucleotides. Antiviral Res. 1:95.

Stuart-Harris, C.H., Schild, G.C. and Oxford, J.S. 1985. 'Influenza: The virus and the disease'. 2nd edition. Publ. Arnold, London.

Szweczuk, M.R. and Mukkur, T.K.S. 1977. Enthalpy-entropy compensation in dinitrophenyl-anti-dinitrophenyl antibody interaction(s). Immunology 32:11.

Tainer, J.A., Getzoff, E.D., Paterson, Y., Olson, A.J. and Lerner, R.A. 1985. The atomic mobility component of protein antigenicity. Ann Rev. Immunol. 3:501.

Tashiro, M., Cibrowski, P., Klenk, H.-D., Pulverer, G. and Rott, R. 1987a. Role of Staphylococcus protease in the development of influenza pneumonia. Nature (Lond.) 325:536.

Tashiro, M., Cibrowski, P., Reinacher, M., Pulverer, G., Klenk, H.-D. and Rott, R. 1987b. Synergistic role of Staphylococcal proteases in the induction of influenza virus pathogenicity. Virol. 157:421.

Taylor, H.P. 1986. PhD thesis, University of Warwick.

Taylor, H.P., Armstrong, S.J. and Dimmock, N.J. 1987. Quantitative relationships between an influenza virus and neutralising antibody. Virol. 159: in press.

Taylor, H.P. and Dimmock, N.J. 1985a. The mechanism of neutralisation of influenza virus by sIgA is different from that of monomeric IgA or IgG. J. Exp. Med. 161:198.

Taylor, H.P. and Dimmock, N.J. 1985b. Mechanisms of neutralisation of influenza virus by IgM. J. Gen. Virol. 66:903.

Thomas, A.A.M., P. Brioen, and A. Boeye. 1985. A monoclonal antibody that neutralises poliovirus by cross-linking virions. J. Virol. 54:7.

Thomas, A.A.M., Vrijsen, R. and Boeye, A. 1986. Relationship between poliovirus neutralisation and aggregation. J. Virol.

59:479.

Tian, S.-F., Buckler-White, A.J., London, W.T., Reck, L.J., Chanock, R.M. and Murphy, B.R. 1985. Nucleoprotein and membrane protein genes are associated with restriction of replication of influenza A/mallard/NY78 virus and its reassortants in squirrel and monkey respiratory tracts. J. Virol. 53:771.

Tobita, K and Kilbourne, E.D. Genetic recombination for antigenic markers of antigenically different strains of influenza B virus. J. Virol. 13:347.

Tomasi, T.B. Jnr. 1976. 'The immune system of secretions'. Publ. Prentice Hall, New Jersey.

Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature (Lond.) 302:575.

Tversidov, V.A., Karadaghi, S.E., Bucher, D.J., Zakomirdin, J.A. and Kharitonenkov, I.G. 1984. Interaction of influenza virus proteins with planar bilayer lipid membranes. II Effects of rimantadine and amantadine. Biochim. Biophys. Acta 778:276.

Ulmanen, I., Broni, B.A. and Krug, R.M. 1981. The role of two of the influenza core P proteins in recognising cap I structures (M^7 GpppNm) on RNAs and in initiating viral RNA transcription. Proc. Natl. Acad. Sci. U.S.A. 78:7355.

Ulmanen, I., Broni, B.A. and Krug, R.M. 1983. Influenza virus temperature sensitive cap M^7 GpppNm dependent endonuclease. J. Virol. 45:27.

Underwood, P.A., Skehel, J.J. and Wiley, D.C. 1987. Receptor-binding characteristics of monoclonal antibody selected antigenic variants of influenza virus. J. Virol. 61:206.

Valentine R.C. and Green, R.M. 1967. Electron-microscopy of an antibody-hapten complex. J. Mol. Biol. 27:615.

van Meer, G., Davoust, J. and Simons, K. 1985. Parameters affecting low-pH-mediated fusion of liposomes with the plasma membrane of cells infected with influenza virus. Biochemistry 24:3593.

van Regenmortel, M.H.V. 1986. Which structural features determine protein antigenicity? TIBS 11:36.

van Regenmortel, M.H.V. 1987. Antigenic cross-reactivity between proteins and peptides: new insights and applications. TIBS 12:237.

van Regenmortel, M.V.H. and Neurath, A.R. 1985. Structure of viral antigens. In 'Immunochemistry of viruses' eds. as above. Publ. Elsevier, Amsterdam, New York and Oxford. p.1.

van Wyke, K.L., Bean Jr., W.J. and Webster, R.G. 1981. Monoclonal antibodies to the influenza A virus nucleoprotein affecting RNA transcription. J. Virol. 39:313.

van Wyke, K.L., V.S. Hinshaw, W.J. Bean, and R.G. Webster. 1980. Antigenic variation of influenza A virus nucleoprotein detected with monoclonal antibodies. J. Virol. 35:24.

van Wyke, K.L., Yewdell, J.W., Reck, L.J. and Murphy, B.R. 1984. Antigenic characterisation of influenza A virus matrix protein with monoclonal antibodies. J. Virol. 49:248.

Varghese, J.N., Laver, W.G. and Colman, P.M. 1983. Structure of the influenza virus antigen neuraminidase at 2.9 A resolution. Nature (Lond.) 303:35.

Wallis, C. and Melnick, J.L. 1967. Virus aggregation as the cause of the non-neutralisable persistent fraction. J. Virol. 1:478.

Wang, M.-L., Skehel, J.J. and Wiley, D.C. 1986. Comparative analyses of the specificities of anti-influenza HA antibodies in human sera. J. Virol. 57:124.

Ward, C.W., and Dopheide, T.A. 1981. Amino acid sequence and oligosaccharide distribution of the haemagglutinin from an early Hong Kong variant A/Aichi/2/68B (X-31). Biochem. J. 193:953.

Webster, R.G., and Laver, W.G. 1967. Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus. J. Immunol. 99:49.

Webster, R.G., and Laver, W.G. 1980. Determination of the number of non-overlapping antigenic areas of Hong Kong (H3N2) influenza virus HA with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virol. 104:139.

Webster, R.G., Hinshaw, V.S. and Laver, W.G. 1982. Selection and analysis of antigenic variants of the neuraminidase N2 influenza viruses with monoclonal antibodies. Virol. 177:93.

Webster, R.G., Brown, L.E. and Jackson, D.C. 1983. Changes in the antigenicity of the HA molecule of H3 influenza virus at acidic pH. Virol. 126:587.

Werner, T.C., Bunting, J.R. and Cathou, R.E. 1972. The shape of immunoglobulin molecules in solution. Proc. Natl. Acad. Sci. U.S.A. 69:795.

Wetz, K., Willingmann, P., Zeichhardt, H. and Habermehl, K.-O. 1986. Neutralisation of poliovirus by polyclonal antibodies requires binding of a single IgG molecule per virion. Arch. Virol. 91:207.

Wharton, S.A., Skehel, J.J. and Wiley, D.C. 1986. Studies of influenza HA-mediated membrane fusion. Virol. 149:27.

White, J., Helenius, A. and Gething, M.-J. 1982. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature (Lond.). 300:658.

White, B.A. and Bancroft, F. 1982. Cytoplasmic dot hybridisation. Simple analysis of relative mRNA levels in multiple small

cell or tissue samples. J. Biol. Chem. 257:8569.

WHO Memorandum. 1980. A revision of the system of nomenclature for influenza viruses. Bull. W.H.O. 58:585.

Wiley, D.C., Wilson, I.A. and Skehel, J.J. 1981. Structural identification of the antibody-binding sites of Hong Kong influenza HA and their involvement in antigenic variation. Nature (Lond.). 289:373.

Wiley, D.C. 1983. Neuraminidase of influenza virus reveals a flower-like head. Nature (Lond.) 303:19.

Williams, A.F. 1984. The immunoglobulin superfamily takes shape. Nature (Lond.) 308:12.

Wiliams, J.C. and Bell, R.M. 1982. Membrane matrix disruption by melittin. Biochim. Biophys. Acta 288:255.

Williams, R.J.P. and Moore, G.R. 1985. Protein antigenicity, organisation and mobility. TIBS 10:96.

Wilson, I.A., Skehel, J.J. and Wiley, D.C. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature (Lond.) 289:366.

Winter, G. and Fields, S. 1980. Cloning of influenza cDNA into M13. The sequence of the RNA segment encoding the A/PR/8/34 matrix protein. Nucleic Acids Res. 8:1965.

Winter, G. and Fields, S. 1981. The structure of the gene encoding the nucleoprotein of human influenza virus A/PR/8/34. Virol. 114:423.

Witt, D.J., Naeve, C.W., and Summers, D.F. 1981. Phosphorylation of VSV virus proteins as a possible contributing factor in virion uncoating. J. Gen. Virol. 56:383.

Wohlfart, C.E.G., Svensson, U.K. and Everitt, E. 1985. Interaction between HeLa cells and adenovirus type 2 virions neutralised by different antisera. J. Virol. 56:896.

Wolstenholme, A.J., Barret, T., Nichol, S.T. and Mahy, B.W.J. 1980. Influenza virus-specific RNA and protein synthesis in cells infected with temperature-sensitive mutants defective in the genome segment encoding non-structural proteins. J. Virol. 35:1.

Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. Analytical Biochemistry 118:197.

Wrigley, N.G. 1979. Electron microscopy of influenza virus. Brit. Med. Bull. 35:35.

Wrigley, N.G., Brown, E.B., Daniels, R.S., Skehel, J.J. and Wiley, D.C. 1983b. Electron microscopy of influenza virus HA-monoclonal antibody complexes. Virol. 131:308.

Wrigley, N.G., Brown, E.B. and Skehel, J.J. 1983a. Electron microscopic evidence for the axial rotation and interdomain

flexibility of the Fab regions of immunoglobulin G. J. Mol. Biol. 169:771.

Wrigley, N.G., Laver, W.G. and Downie, J.C. 1977. Binding of antibodies to isolated HA and NA molecules of influenza virus observed in the electron microscope. J. Mol. Biol. 109:405.

Wrigley, N.G., Skehel, J.J., Charlwood, P.A. and Brand, C.M. 1973. The size and shape of influenza virus neuraminidase. Virol. 51:525.

Yamakawa, M., Furuichi, Y., Nakashima, F., La Fiandra, A.J. and Shatkin, A.J. 1981. Excess synthesis of viral mRNA 5' terminal oligonucleotides by reovirus transcriptase. J. Biol. Chem. 256:6507.

Ye, Z., Pal, R., Ogden, J.R., Snyder, R.M. and Wagner, R.R. 1985. Monoclonal antibody to the matrix protein of VSV (New Jersey serotype) and their effects on viral transcription. Virol. 143:657.

Yewdwell, J.W., Caton, A.J. and Gerhard, W. 1986. Selection of influenza A virus adsorptive mutants by growth in the presence of a mixture of monoclonal anti-HA antibodies. J. Virol. 57:623.

Yewdell, J.W., Gerhard, W. and Bachi, T. 1983. Monoclonal antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/834 mediated haemolysis. J. Virol. 48:239.

Yewdell, J.W., Webster, R.G. and Gerhard, W. 1979. Antigenic variation in three distinct determinants of an influenza type A haemagglutinin molecule. Nature (Lond.) 279:246.

Yoden, S., Kida, H. and Yanagawa, R. 1982. An avian influenza virus of which infectivity is neutralised by antisera lacking haemagglutination inhibition activity. Arch. Virol. 74:205.

Yoden, S., Kida, H. and Yanagawa, R. 1985. Is bivalent binding of monoclonal antibody to different antigenic areas on the HA of influenza virus required for neutralisation of viral infectivity? Arch. Virol. 85:209.

Yoshida, T.M., Shaw, M., Young, J.F. and Compans, R.W. 1981. Characterisation of the RNA associated with influenza A cytoplasmic inclusions and the interaction of NS1 protein with RNA. Virol. 110:87.

Yoshimura, A., Kuroda, K., Kawasaki, K., Yamashina, S., Maeda, T. and Ohnishi, S.-I. 1982. Infectious cell entry mechanism of influenza virus. J. Virol. 43:284.

Yoshimura, A., and Ohnishi, S.-I. 1984. Uncoating of influenza virus in endosomes. J. Virol. 51:497.

Young, R.J. and Content, J. 1971. 5' terminus of influenza virus RNA. Nature New Biol. 230:140.

Zavada, J. 1982. The pseudotype paradox. J. Gen. Virol. 63:15.

Zebedee, S.L., Richardson, C.D. and Lamb, R.A. 1985. Characterisation of the influenza virus M2 integral membrane protein and expression at the infected-cell surface from cloned cDNA. J. Virol. 56:502.

Zhirnov, O.P. and Bukrinskaya, A.G. 1981. Two forms of influenza virus nucleoprotein in infected cells and virions. Virol. 109:174.

Zhirnov, O.P., Ovcharenko, A.V. and Bukrinskaya, A.G. 1984. Suppression of influenza virus replication in infected mice by protease inhibitors.

Zvonarjev, A.Y., and Ghendon, Y.Z. 1980. Influence of membrane (M) protein on influenza A virus virion transcriptase activity in vivo and its susceptibility to rimantadine. J. Virol. 33:583.